IMMUNOLOGICAL AND STRUCTURAL COMPARISONS OF THE POKEWEED ANTIVIRAL PROTEINS FROM *PHYTOLACCA RIGIDA* AND DERIVED CALLUS TISSUE

BY

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LIST OF ABBREVIATIONS

A_{280}	absorbance at 280 nanometers
A_{405}	absorbance at 405 nanometers
BSA	bovine serum albumin
crm	cross-reactive material
ddw	deionized distilled water
EITB	western blot
ELISA	enzyme linked immunosorbent assay
h	hour(s)
IC-50	inhibitory concentration -50% value of control level
kDa	kilodalton
uL	microliter(s)
ug	microgram(s)
mg	milligram(s)
min	minute(s)
mL	milliliter(s)
mRNA	messenger RNA
ng	nanogram(s)
PAP	pokeweed antiviral protein
PBS	phosphate buffered saline
PBS:Tw	phosphate buffered saline containing 0.3% (v/v)Tween-20
PBS:Tw:BSA	PBS:Tw with 0.1% BSA
RIP	ribosome-inactivating protein

rRNA	ribosomal RNA
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
TBS	Tris buffered saline
v.s.	vide supra

Abstract of Dissertation Presented to the Graduate School of the University of Florida in Partial Fulfillment of the Requirements for the Degree of Doctor of Philosophy

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Phytolacca rigida (common 'Southern' pokeweed) has been shown to possess three proteins, PAP-I (spring leaf), PAP-II (summer leaf) and PAP-S (seed), which are potent inactivators of eukaryotic ribosomal function. These proteins are presumed to be separate gene products and are developmentally regulated with respect to their biosynthesis and tissue localization. They are all very basic proteins with similar pI's, molecular masses, and with significant amino acid homology (sufficient homology to predict common evolutionary lineage). As inhibitors of in vitro translation they are equivalent, acting as N-glycosidases removing the A4324 from the 28S rRNA on rat liver and rabbit reticulocyte ribosomes. These proteins have collectively been termed ribosome-inactivating-proteins and are widespread throughout the plant kingdom. This research was undertaken to prepare antibody probes against the three PAP proteins for immunological comparisons, to develop a tissue culture system for the expression of the PAP-proteins and to utilize the antibody preparations as probes for the PAP proteins in leaf or tissue

culture. Utilizing the antibody probes it was demonstrated that PAP-I is immunologically cross-reactive with both PAP-II and PAP-S, but that PAP-II and PAP-S show limited cross-reactivity. The tissue culture system was found to express a protein, detected with monospecific anti-PAP-I, which was the equivalent of the PAP-I protein from spring leaf tissue. Monospecific anti-PAP-II identified cross-reactive material which was of larger molecular weight, glycosylated and possessed a unique amino terminal sequence when compared to the leaf form. Monospecific antibodies to PAP-I and to PAP-II were utilized in immunocytochemical studies to localize the proteins in tissue culture. Antibody was developed against a unique amino terminal sequence associated with the anti-PAP-II cross-reactive material and utilized as a probe of crude leaf extracts for proteins which were immuno-reactive. In developmental studies PAP-I and PAP-II were shown to be co-synthesized in leaf tissue. The antibody probe against the unique amino terminal sequence detected discrete molecular entities which may correspond to processing precursors for the PAP-II protein.

CHAPTER I
INTRODUCTION

Historical Perspective and Comparative Properties of Ribosome-Inactivating Proteins

Proteins which are toxic to cells (mammalian or otherwise) are found widely distributed throughout Nature. Those found in sufficient quantity to study are frequently obtained from plants. Two plants, Ricinus communis and Abrus precatorius have a long history of medicinal use and it was early recognized that they contained one or more toxic principles. The toxic moieties were characterized in the late nineteenth century as proteins. These two proteins, abrin (from Abrus precatorius) and ricin toxin (from Ricinus communis) represent the first described toxic proteins from plants and they have been extensively studied (Olsnes and Pihl, 1976). Lin et al. (1970) demonstrated that both toxins have the same effect on mammalian cells in culture, i.e., they are both comparable and potent inhibitors of protein synthesis. Later investigations established that these proteins are heterodimers, consisting of an A chain and a B chain. It was found that in the presence of a reducing agent (e.g. mercaptoethanol) the two chains could be separated and that the reduced toxin was no longer toxic to intact cells but had the ability to inhibit in vitro cell-free protein synthesis. When tested separately, it was demonstrated that only the toxin A chain could inhibit protein synthesis in a cell-free system and that the B chain was without effect. The B chain was shown to possess agglutinating characteristics and served a binding function for the intact toxin. The B chain

acts as a lectin and either free or linked to the A chain by a disulfide bridge preferentially binds to terminal galactose residues. This activity is responsible for promoting the association of the holotoxin with cell surface glycoproteins which may promote endocytosis. The A chain was shown to possess a catalytic enzymatic activity which inactivated the 60S ribosomal subunit of eukaryotic ribosomes. This activity is associated with a reduction both in the binding of elongation factor EF-1 to the ribosomes and EF-2 dependent GTP-ase activity (Fernandez-Puentes and Vazquez, 1977; Obrig et al., 1973). Because of the carbohydrate binding properties of the B chain and the toxic effects of the A chain these proteins have been termed cytotoxic lectins.

Since the original studies on abrin and ricin, a large number of proteins from plants have been identified which share the ability to inhibit protein synthesis in vitro and in vivo. Historically, these proteins were initially termed ribosome-inhibiting proteins (Obrig et al., 1973), until it was demonstrated that they irreversibly inhibited ribosome function. This irreversible inhibition is effected through the N-glycosidase cleavage of a specific adenine residue in 28S rRNA resulting in inactivation of the ribosome (Endo et al., 1987). Because of their ability to inactivate the eucaryotic ribosome these proteins have been more appropriately termed 'ribosome-inactivating proteins' or RIPs (Barbieri and Stirpe, 1982). Many of these proteins are monomeric polypeptides which are functional analogs of the ricin A chain. In order to distinguish the heterodimeric from the monomeric proteins, the heterodimers are termed Type 2 RIPs, and the monomers have been classified as Type 1 RIPs (Stirpe and Barbieri, 1986).

Interestingly, this class of proteins (Type 1 RIPs) is found within a large number of different plant families and these proteins may be a ubiquitous

feature of all plants (Gasperi-Campani et al.,1985). However, even though these proteins are found widely distributed throughout the plant kingdom, there are significant differences in their absolute amounts between families and orders. In particular, several families within the order Centrospermae include species which show high levels of these proteins. In large scale screening studies of crude extracts from plants representing many orders it was found that proteins (crude extracts) from plants within the Centrospermae may be 10 to 100-fold more potent than proteins from plants outside the Centrospermae (Grasso and Shepherd, 1978). Within the Centrospermae the highest concentrations are found in plants represented by the families of Caryophyllaceae, and the Phytolaccaceae. Outside the Centrospermae, two other orders have members which show high levels of these proteins. In particular, the Cucurbitaceae (cucumber family in the order Violales) and members of the order Euphorbiales include species which are rich in these proteins. Ricinus communis (from which ricin toxin has been isolated) is a member of the Euphorbiales. As more information becomes available it appears that these proteins are more common to members of the Caryophyllaceae, Phytolaccaceae and Cucurbitaceae which contain greater amounts of these proteins than do members of the Euphorbiaceae. Table 1.1 relates the taxonomic distributions and some salient biophysical properties of RIPs in those plant species found to contain significant amounts of monomeric ribosome-inactivating proteins. These proteins are also represented in the Gramineae (grass family) of which those from Triticum (wheat), Secale (rye) and Hordeum (barley) have been the most well characterized (Coleman and Roberts, 1982; Asano et al.,1984,1986). The proteins have been isolated from a variety of structures including seeds, roots, leaves and latices. However, within an individual

Table 1.1 Properties of characteristic Type-1 ribosome-inactivating proteins

Taxonomy		Biophysical Characteristics			
Family	Trivial name	Tissue Locale	Mr ($\times 10^{-3}$)	% CH_2O	pI
Caryophyllaceae					
Dianthus[a] *caryophyllus*	dianthin 30	leaf	29.5	1.6	8.7
	dianthin 32	leaf	31.7	2.3	8.5
Saponaria[b] *officinalis*	saporin-6	seed	29.5	0	>9.5
Agrostemma[b] *githago*	agrostin-2	seed	30.6	7.0	7.7
	agrostin-5	seed	29.5	7.0	>8.0
	agrostin-6	seed	29.6	7.0	>8.0
Cucurbitaceae					
Bryonia[c] *dioica*	bryodin	root	30.0	6.3	>9.5
Cucumis[d] *melo*	cucumin	seed	23.5	nr[j]	nr
Luffin[e] *cylindrica*	luffin	seed	26.0	nr	nr
Momordica[a] *charantia*	momordin (MCI)	seed	31.0	1.7	8.6
Trichosanthes[f] *kirilowii*	trichosanthin	root	26.5	0	9.4
Trichosanthes[g] *kirilowii*	trichokirin	seed	27.5	1.3	>9.0

Table 1.1(continued)

Taxonomy		Biophysical Characteristics			
Family	Trivial name	Tissue Locale	Mr ($x10^{-3}$)	% CH_2O	pI
Euphorbiaceae					
Gelonium[a] multiflorum	gelonin	seed	30.0	4.5	8.2
Hura[b] crepitans	---	latex	28.0	40.0	nr[j]
Graminae					
Hordeum[h] vulgare	BPI	seed	30.0	nr	nr
Secale[i] ---	---	seed	30.0	nr	nr
Triticum[i] aestivum	tritin	seed	30.0	nr	nr
Liliaceae					
Asparagus[b] officinalis	peak 2 peak 3 peak 5	seed seed seed	32.5	1.4	>9.5

a. Falasca et al., 1982; b. Stirpe et al., 1983; c. Stirpe et al., 1986; d. Ferreras et al., 1989;
e. Kishida et al., 1983; f. Maraganore et al., 1987; g. Casellas et al., 1988;
h. Asano et al., 1984; i. Coleman and Roberts, 1982; j. nr- no report

plant species they are usually found uniquely concentrated within a specific tissue. As is apparent from Table 1.1, however, these proteins are most frequently found associated with seed tissue (as is also the case for the Type 2 toxins, abrin and ricin) and/or leaf tissue.

Comparison of Pokeweed Antiviral Proteins and Other Ribosome-Inactivating Proteins

The ribosome-inactivating proteins from Phytolacca species have been well studied. In particular, the proteins from Phytolacca americana have served as a source of study since the original observations of Duggar and Armstrong (1925) suggested these proteins to possess an antiviral activity. In this initial study the researchers demonstrated that the sap from P. americana was able to inhibit viral transfer between tobacco and P. americana. This ability to inhibit viral transfer was identified prior to the characterization of the active principle as a protein. Several attempts made to define the active principle in the plant extracts capable of causing viral inhibition established the material as a protein (Kassanis and Kleczkowski,1948) with a minimum molecular weight of 13,000 (Wyatt and Shepherd, 1969). These observations resulted finally in the purification and characterization of the active moiety in spring leaf tissue as a basic protein with the catalytic property of inactivating eukaryotic ribosomes (Obrig et al.,1973; Irvin, 1975). Subsequently, proteins with similar properties to those ascribed to PAP-I (spring leaf tissue) were isolated from the summer leaf tissue (PAP-II, Irvin et al.,1980) and the seeds (PAP-S, Barbieri et al.,1982) of P. americana. There is reference to a PAP-R (from roots) but the properties of that protein have not been reported (Cenini et al., 1988). The PAP proteins isolated from these various tissues share several physical

Table 1.2 Properties of ribosome-inactivating proteins associated with Phytolacca spp. and callus tissue.

Taxonomy		Characteristics			
Family	Trivial name	Tissue Locale	Mr ($\times 10^{-3}$)	% CH_2O	pI
Phytolaccaceae					
Phytolacca americana	PAP-I[a]	leaf	29.5	0	8.1
	PAP-II[b]	leaf	30.5	0	8.3
	PAP-S[c]	seed	30.0	0	8.5
P. dodecandra	Dodecandrin[d]	leaf	29.5	0	>8.1
P. rigida	PAP-1[e]	leaf	29.5	0	>8.0
	PAP-II[e]	leaf	30.5	0	>8.0
	PAP-S[e]	seed	30.0	0	>8.0
P.americana callus	PAP-C[f]	callus	22.0-29.0	0	>8.0
P.rigida callus	Peak 4[g]	callus	29.5	0	>8.0
	Peak 1[g]	callus	44.0	19.4	>8.0
	Peak 2[g]	callus	34.0	13.2	>8.0

a. Irvin et al., 1975
b. Irvin et al., 1980
c. Barbieri et al., 1982
d. Ready et al., 1984
e.Preston and Ervin, 1987
f. Barbieri et al., 1989
g. Ervin and Preston, 1988

and biological properties. They are all highly basic proteins with published pI values of greater than 8.0, and similar molecular weights of about 30,000. Most importantly, they are all equally effective as inhibitors of in vitro translation, indicating that they share the same enzymatic activity. All members of the Phytolaccaceae so far investigated (to this date restricted to Phytolacca spp.) possess a ribosome-inactivating protein analogous to those described from P. americana. Table 1.2 shows the biophysical properties of those members of the Phytolaccaceae known to possess a RIP, and for comparison the properties of the callus tissue proteins described in the present research.

It is obvious from the data compiled in Tables 1.1 and 1.2 that the RIPs comprise a large family of related proteins. While the overall similarities at the biophysical level relate these proteins one to the other, the available amino acid sequence data reveal differences which belie the overall relatedness of these proteins. To demonstrate this point amino acid sequence data for selected RIPs are presented in Table 1.3.

Inspection of the sequence data presented in Table 1.3 reveals little homology in sequence between proteins from different taxonomic families, while within families one sees good homology. This observed difference in sequence between proteins which share extensive biophysical similarities may reflect amino acid changes which do not affect the overall tertiary structure of the proteins (Montecucchi et al., 1989).

In the case of the ribosome-inactivating proteins, the complete primary structure (amino acid sequence) was first obtained for ricin A chain (RTA) (Funatsu et al., 1979; Yoshitake et al., 1978) and the gene encoding a ricin precursor was isolated, cloned and sequenced by Lamb et al. (1985).

Table 1.3 Amino terminal sequence comparisons between selected RIPs, including PAP-like proteins from Phytolacca callus tissue.

Toxin Family Common Name	Sequence
Euphorbiaceae	
RTA[a]	IFPK QYPI INFI IAGAIVQSYINF IRAV
Gelonin[a]	GLDIVSFSIKGAIY II YVNFLNEL
Caryophyllaceae	
SO-6[a]	VISIILDLVNPIAGQYSSFV DKIR
Phytolaccaceae	
Dodecandrin[a]	VNTIIYNVGSI TISNYATFMDNIR
PAP-I[a] (P.amer.	VNTIIYNVGSI TISKYATFLNDLR
PAP-I[b] (P.rigid.)	data not available
PAP-S[a] (P.amer.)	INIITFDAGHATI NKYATFY ESLX
PAP-S[b] (P.rigid.)	I N/D I I T F D A G
PAP-II[a](P.amer.)	- N - I VFDVE N AIPEIYSNFLI SLR
PAP-II[b] (P.rigid.)	N/D - I VFDVG N/D A I
PAP-C[c] (P.amer.)	VN I I IYNVGS
Pk4 Callus[d](P.rigid.)	V - I AIYI- - - (incomplete sequence)
Cucurbitaceae	
BD[a]	DVSFRLSGADPRSYGMFIKDLRNA
TK[a]	DVSFRLSGAISSSYGVFISNLRKA
MC[a]	DVSFRLSGADPRSYGMFIKDL
Graminae	
BPI[e]	AAKMAK NVDKPLFIAIFNVQASSADYA

Footnotes:
a. Montecucchi et al., 1989
b. unpublished data obtained from Protein Core Facility, Univ. Fl
c. Barbieri et al. 1989
d. Ervin, 1989 (this work)
e. Asano et al., 1986
f. N/D signifies an ambiguous amino acid at that position, the preferred amino acid is signified first.

Trichosanthin (from Trichosanthes kirilowii, Xuejun and Jiahuai, 1986) and the barley protein inhibitor (BPI, Asano et al., 1986) from Hordeum vulgare have recently been completely sequenced as pure proteins. Incomplete amino terminal sequence data are available for a large number of RIPs, (both Type1 and Type 2) including PAP-I, PAP-II, PAP-S and dodecandrin (from Phytolacca spp.) and the A chain from modeccin (Adenia digitata, Ready et al., 1984). Sequence data for saporin 6 (SO-6, from Saponaria officinalis) have been reported from DNA sequencing of a gene isolated and cloned from leaf tissue which encodes a seed-like protein (Benatti et al., 1989). Based on sequence data homology to RTA and known biophysical and functional similarities, all of the ribosome-inactivating proteins are hypothesized to have evolved from a common ancestral gene (Ready et al., 1988). The availability of the gene sequence and the long history of study of ricin culminated in the crystallographic imaging of that protein at 2.8 A resolution (Montfort et al.,1987). Based on these imaging studies, as well as site directed mutagenesis studies (Hovde et al., 1988) and amino terminal sequence data obtained from a number of RIPs revealing five highly conserved amino acids (Montecucchi et al., 1989), a picture of the active site of these proteins has begun to emerge (Robertus, 1988).

In the early period of study of these proteins, prior to the detailed crystallographic data now available for RTA, chemical modifications of the PAP-I protein were performed to gain some understanding of the requirements for the enzymatic activity of the protein. Such treatments as titration of -SH groups with dithiothreitol and treatments with diethylpyrocarbonate or phenyl glyoxal revealed some aspects of the amino acid structures in the microenvironment of the active site (see Irvin, 1983 for review). Such studies by chemical modification yield information which is

distinct from that obtained with proteolytic digests. For the single chain RIPs, in particular those from Phytolacca, there is limited structural data based on chemical or enzymatic proteolysis.

Trypsin has long been a favorite tool for structural analysis as its mode of action is well defined and tryptic maps of peptides obtained from enzymatic digests are frequently quite repeatable. Historically it had been observed that the RIPs (both Type 1 and Type 2) are resistant to enzymatic proteolysis. In particular, it was reported that intact ricin or abrin (and by extension, other RIPs) are insensitive to treatment with proteolytic enzymes. However, the isolated A or B chains are susceptible, with the A chain being more sensitive (Olsnes and Pihl, 1982). Stirpe et al. (1983, 1986) were unable to affect the enzymatic activity of bryodin or SO-6 by incubation with trypsin, chymotrypsin or subtilisin at a 0.01 molar ratio of enzyme to RIP. The SO-6 seed protein from Saponaria, however, was cleaved with clostripain to give small peptide fragments suitable for sequencing from which oligonucleotide probes were synthesized (Benatti et al, 1989). From the preparation of tryptic maps, it was demonstrated that ricin A chain and the related agglutinin A chain were closely related (Olsnes and Pihl, 1976). From the analysis of peptide maps prepared from tryptic digests of PAP -I and PAP-II, Irvin et al. (1980) concluded that few, if any of the peptides could be considered to be homologous between PAP-I and PAP- II.

Other Properties of Phytolacca spp.

In addition, the Phytolacca spp. are a source for the pokeweed mitogens, which are capable of differentially stimulating DNA synthesis of B and/or T cells of murine and human origin. These mitogens have found extensive use in immunological studies (Waxdal et al., 1976). The species native to North Africa, P. dodecandra (endod) has been shown to possess a protein

which is nearly equivalent to the PAP-I from P. americana (Ready et al., 1984). This plant also has found utility as a specific molluscide in the control of schistosomiasis, due to the saponins found in the stem and berries (Kloos, 1979). The presence of these other metabolites appears to be unrelated to the presence of the ribosome-inactivating proteins in these plants. However, these unique chemical properties of Phytolacca prompted the Korean researchers Misawa et al. (1975) and Woo and Kang (1976) to develop a tissue culture system to study the production of these compounds.

Due to their catalytic activity and mode of action (v.s.), the toxic proteins ricin and abrin (Lin et al., 1970) were first considered as potential anti-tumour agents (Lord, 1987). The identification of analogous biophysical properties and enzymatic activities of PAP-I and PAP-S with those of ricin A chain led to the early evaluation of the PAP forms in preparing specific immunotoxins. The non-specific binding of the Type 2 toxins (abrin, ricin, modeccin) mediated by the B chain made it difficult to work effectively with these toxins in vivo. However, the construction of antibody conjugates with either free A chain from ricin or abrin, or with Type 1 toxins which possessed only the A chain offered the potential of greater targetting specificity. The efficacy of such toxin-antibody immuno-conjugates was demonstrated for PAP-I by Masuho et al. (1982). Much research has been fostered in this area and it continues to be an area of great activity (Frankel, 1988). In addition to their application as cell-specific cytotoxic agents in immuno-conjugates, many of the RIPs have been shown to possess an abortifacient activity (Yeung et al., 1988). In at least one case (trichosanthin) extracts from the plant bearing the RIP have been used medicinally as an abortifacient for hundreds of years (Kuo-Fen, 1982). The activity as an abortifacient has been shown to be related to the RIP present in the tuber (Maraganore et al.,

1987). The applications in medical sciences of the RIPs are all related to the unique enzymatic activity of these proteins.

Ribosome-Inactivating Proteins Associated with Plant Toxins

As mentioned previously, the ability to inactivate eukaryotic ribosomes was originally found in a group of proteins termed cytotoxic lectins. Due to the presence of a B chain, these proteins are frequently non-specific in their cytotoxicity towards mammalian cells. These proteins are exemplified by ricin which is isolated from the seeds of the castor bean plant (from Ricinus communis) . The most well studied of this class of heterodimeric toxins includes abrin (from Abrus precatorius) and modeccin (from Adenia spp.). (see Olsnes and Pihl, 1982 for a review).

Ricin toxin is a heterodimeric glycoprotein with an apparent molecular weight of 65 kD. The two polypeptide chains are bound covalently by a single disulfide bond. The B chain is specific for terminal galactose residues and is able to bind to cell surface glycoproteins by virtue of this property. The bound toxin, A and B chains linked by a disulfide bond is internalized and by an ill-defined process the undegraded A chain is delivered across the vesicular membrane and into the cytosol. The A chain, which is analogous to the monomeric toxins described earlier, is capable of catalytically inactivating the 60S ribosomal subunit RNA.

Mechanism of Action

The mechanism of action of these toxins was the subject of much research in the last two decades but was finally resolved by the work of Endo et al. (1987). These researchers demonstrated that the ricin A chain deadenylated a specific residue in the 28S ribosomal RNA from rat liver reticulocytes. The mechanism of action was described as the hydrolysis of

the N-glycosidic bond between the adenine and ribose at residue A4324 in the ribosomal 28S RNA (Endo and Tsurugi, 1987). The A chain then is an N-glycosidase with a very specific substrate. It was soon demonstrated that many of the proteins of this sort, capable of inhibiting in vitro translation, shared this same mechanism of action (Endo et al., 1988; Stirpe et al.,1988). Similar effects were seen with a recombinant ricin A chain on yeast ribosomes (Bradley et al., 1987). It is interesting to note that the bacterial toxin from Shigella dysenteriae 1 (Shiga toxin, Hovde et al., 1988) and that from enterohemmorrhagic Escherichia coli (Vero toxin), share this same mechanism of inactivation of the 28S rRNA (Endo et al., 1988)

Ii is now known that the fungal toxin, alpha-sarcin from Aspergillus giganteus , is capable of the specific cleavage of the phosphodiester bond between the guanosine residue at position 4325 and the adenosine residue at position 4326 in the 28S rRNA from yeast or wheat germ (Endo and Wool, 1982). Alpha-sarcin then is a phosphodiesterase whose site of cleavage is one nucleotide removed from that of the RIPs. Alpha-sarcin preferentially cleaves the large rRNA from ribosomes of rat, Xenopus, yeast, Zea chloroplast, mouse mitochondria and human mitochondria and E. coli (Wool, 1984). In contrast to this broad range of susceptible rRNA's known for alpha-sarcin, the RIPs show a greater specificity. In general, mammalian ribosomes are susceptible, as are those from yeast. Wheat germ is an effective substrate for the PAPs but not apparently for ricin A chain (Preston and Ervin, 1987). Ricin (and by analogy, other RIPs) does not affect ribosomes from E. coli nor from rat liver mitochondria (Greco et al., 1974). In addition, ribosomes from Sulfolobus (Archaebacterium) are insensitive to the action of dianthin-32, gelonin, momordin, PAP-I and ricin A-chain (Cammarano et al., 1985). Type 1 RIPs are differentially effective against the

isolated ribosomes from the protozoa Acanthamoeba , Tetrahymena, Leishmania and Trypanosoma, while Type 2 RIPs are virtually ineffective (Cenini et al., 1988; Cenini et al., 1987).

While this differential susceptibility of mammalian, protozoan and bacterial ribosomes might be expected because of the presence of unique associated ribosomal proteins, different assay conditions or other unknown factors, the situation with ribosomes from plants is even less clear. The early observations of Owens et al. (1973) on pokeweed showed that PAP-I inhibited in vitro translation by wheat germ and cowpea ribosomes but not with pokeweed ribosomes. Coleman and Roberts (1982) showed that the RIP from wheat germ does not affect wheat germ ribosomes. These studies provided the basis for the general hypothesis that RIPs should act only on the heterologous ribosomes (Stirpe and Barbieri, 1986). This problem was adressed in a paper by Battelli et al. (1984) in which the response of ribosomal preparations from a number of RIP producing plants was evaluated against a number of RIPs, including the homologous RIP. Two points emerged from this study. 1) The Type 2 toxins (heterodimers) as ricin, abrin and modeccin are relatively ineffectual inhibitors throughout all preparations assayed and 2) the Type 1 toxins assayed (MCI, dianthin, gelonin, PAP-S) are variable in their ability to inhibit heterologous ribosomes but where assayed show no effect on autologous ribosome preparations. The available data are summarized in Table 1.4 which shows the IC-50 (nM) on susceptible ribosomes (in vitro) and indicates (by an asterisk) those which have been shown to possess an N-glycosidase activity.

Table 1.4. Tabulation of the IC-50's (nM) for the inhibition of cell-free translation by ribosome-inactivating-proteins.

	Ribosome Source								
	Bacterial	Protozoan				Crustacean	Mammalian		
	E.coli	Tetra-hymena	Acantha-moeba	Trypan-osomas	Leish-mania	Artemia	Myeloma	Ascites	R. B Lysate
Toxin									
Type 2									
Abrin*		1700[b]	100[b]	49[c]	5[c]		0.2[l]		0.5-13[i]
Modeccin*		1700[b]	1700[b]	>1700[c]	1700[c]		0.8[l]		2.3[i]
Ricin*	>4200[a]	1700[b]	1000[b]	>1700[c]	>1700[c]	0.20[d]	0.1[l]		0.1[i]
Type 1									
Agrostin									0.5[i]
BPI*				1598[c]	3330[c]				2.1[e]
Bryodin*		3330[b]	430[b]	3330[c]	3330[c]				0.12[q]
Cucumin									0.85[k]
Dianthin 32*		30[b]	7[b]	66[c]	27[c]				0.3[i]
Dodecandrin*			0.08[l]				0.03[l]		0.04[i]
Gelonin*		3300[b]	50[l]-3300[b]	3330[c]	2590[c]	>150[d]	0.20[l]		0.4[d]
Luffin									0.02[h]
MCI*		300[b]	600[l]-3330[c]	>3330[c]	70[j]	0.01[l]	0.20[l]		
PAP-I*			0.8[l]			2.4[m]	0.30[l]		0.24[i]-1.25[o]
PAP-II*									0.25[i]-1.25[o]
PAP-S*		1600[b]	0.4[l]-1230[b]	17[c]	21[c]		0.30[l]		0.08[i]-10[o]
Saporin-6*		2600[b]	0.5[l]-17[b]	116[c]	33[c]		0.04[l]		0.5[i]
Trichosanthin									
Trichokirin*				1265[c]	>3330[c]				0.01[p]
Tritin*						1.0[t]		2.5[n]	8.3[f,i]

Table 1.4 (continued)

Toxin Source	Ribosome Source: Bryonia	Daucus	Dianthus	Nicotiana	Phytolacca	Ricinus	Wheat Germ Extract
Type 2							
Abrin	>3300[r]	3300[r]	>3300[r]	870[r]	>3300[r]	5200[r]	4100[r]
Modeccin	>3300[r]	>3300[r]	>3300[r]	>3300[r]	>3300[r]	>3300[r]	>3300[r]
Ricin	>3300[r]	>3300[r]	>3300[r]	>3300[r]	>3300[r]	>3300[r]	>3300[r]
Type 1							
Agrostin							
BPI							
Bryodin	>3300[q]						
Cucumin							>3300[q]
Dianthin32		385[r]		114[r]	>3300[r]	223[r]	28[r]
Dodecandrin							0.5[s]
Gelonin	820[q]	>3300[r]	>3300[r]	>3300[r]	>3300[r]	>3300[r]	>3300[r]
Luffin							
MCI	>3300[q]	>3300[r]	1200[r]	4200[r]	>3300[r]	>3300[r]	1100[r]
PAP-I							0.03[o]
PAP-II							0.01[o]
PAP-S	12[q]	2100[r]	>3300[r]	80[r]	>3300[r]	1230[r]	0.01[o]-20[q,r]
Saporin-6	8[q]						
Trichosanthin							
Trichokirin							>3300[p]
Tritin							5[n,t]

Footnotes: *-signifies that the protein has been shown to possess an N-glycosidase activity. a. Greco et al., 1974; b. Cenini et al., 1987; c. Cenini et al., 1988; d. Brigotti et al., 1989; e. Asano et al., 1984; f. Reisbig and Bruland, 1983; g. Stirpe et al., 1988; h. Kishida et al., 1983; i. Stirpe and Barbieri, 1986; j. Barbieri et al., 1980; k. Ferreras et al., 1989; l. Villemez et al., 1987; m. Dallal and Irvin, 1978; n. Coleman and Roberts, 1982; o. Preston and Ervin, 1987; p. Casellas et al., 1988; q. Stirpe et al., 1986; r. Batelli et al., 1984; s. Ready et al., 1984; t. Roberts and Selitrennikoff, 1986

While these data may be indicative of a general trend supportive of the interpretation that homologous ribosomes are not affected by the homologous RIP, they do not entirely delimit the boundaries of susceptibility. At the least, it iseems clear that mammalian ribosomes are more sensitive to the action of these toxins than are plant ribosomes, with the exception of those prepared from wheat germ extract. The basis for this differential sensitivity is unknown. An individual toxin may show differential activity towards ribosomes from different sources. Indeed, all the available data indicate a specificity (mediated by unknown factors) in the interaction between ribosomes and RIPs. Battelli et al. (1984) suggest that there are perhaps several distinct populations of ribosomes each differentially susceptible to the action of the autologous toxin. This would support the interpretation that these proteins (RIPs) are involved in the developmental staging and/or regulation of the cell life cycle. However, the cellular protein synthetic machinery would necessarily have to be immune to the action of the toxin unless the toxins were synthesized as an inactive precursor, or sequestered co-translationally away from the cytosol. (v.s.)

Biosynthesis of Lectins and RIPs

Little is known about the biosynthesis of the Type 1 ribosome-inactivating proteins. More is known about the biosynthesis and targetting of the Type 2 toxin , ricin. Ricin toxin is composed of two distinct N-glycosylated subunits, an A chain (M_r 32,000) and a B chain (M_r 34,000) linked by a single disulfide bond. The A and B chains of the toxic lectin are synthesized in the form of a single precursor polypeptide (Butterworth and Lord, 1983). During synthesis this precursor is co-translationally modified on passage into the lumen of the endoplasmic reticulum. These

modifications include the proteolytic cleavage of an amino terminal leader (signal) sequence and glycosylation. The modified precursors are internalized in a second membrane bound compartment during transit to the protein bodies. Within the protein bodies the precursors are proteolytically cleaved (Harley and Lord, 1985) to release the A and B subunits. This mechanism of post-translational precursor cleavage is a sequence common to a number of lectins and plant storage proteins (Chrispeels, 1984; Spencer, 1984).

The Type 1 RIPs are not encumbered by the presence of a B chain during their processing. In addition, the majority of the mature proteins show variable glycosylation. Glycosylation may be an important signal during processing and targetting of proteins (Marshall, 1972). Such is the case for the lectin Concanavalin A (Con A). Con A is first synthesized as an N-linked glycoprotein (Marcus et al., 1984), but the mature protein is not glycosylated. There are precedents for the removal of a glycosylated peptide to yield a mature, non-glycosylated protein . However, in contrast to the proteolytic cleavage of the proricin in the protein body to give the two complete peptide chains, Con A is synthesized from the proteolytic cleavage of a 15 amino acid linker from residue 237 to residue 1. This processing step is followed by a transpeptidation in which the cleavage of an additional nonapeptide from the C terminus occurs simultaneously with the religation between residues 118 and 119 (Sharon and Lis, 1986). This processing sequence has so far been demonstrated only for Concanavalin A . Three important points derive from these observations. 1) The surface loop of 15 amino acids negatively affects the carbohydrate-binding activity of the protein; once the loop is cleaved all precursor forms bind carbohydrate while the form bearing the loop cannot bind carbohydrate. The loop is glycosylated and this may

be important for correct folding. 2) The authors note that "Since the ribosomal-binding proteins of the RER, the ribophorins, are Con-A binding N-glycoproteins, it is improbable that newly synthesized Con-A would ever be transported out of that compartment and to the vacuole if it was in active form." (Bowles and Pappin,1988, p.63) 3) The data imply that there may be only a short, precise time span during development when the function of Con-A is required. Point 2 is potentially important to an understanding of the biosynthesis/targetting of the ribosome-inactivating proteins as, in principle, cytosolic or membrane bound ribosomal RNA should be susceptible to the action of these toxins.

The biosynthetic schema presented above may be relevant to an understanding of the processes operative for the monomeric RIPs. In the two cases discussed briefly above, the final site of deposition of the proteins is known to be in the protein bodies within the seed. While reports for the localization of any of the monomeric RIPs are scanty, PAP-I (spring leaf tissue) has been localized to the cell wall matrix of the P. americana by ferritin immunocytochemistry (Ready et al., 1986). The PAP-I protein could be initially deposited during the formation of the phragmoplast which is the biosynthetic origin of the middle lamella. Alternatively, for PAP-I to be routed to the cell wall during development would require that the protein translocate from the cytosol across the plasma membrane and be deposited as part of the growing wall. This situation is reminiscent of that described for the ricin toxin , although there are some obvious differences. Transport across the plasma membrane and deposition within the polysaccharide rich matrix of the extracellular compartment (cell wall matrix) is not the same as transport across the tonoplast of the vacuolar compartment from which the protein bodies ultimately derive (Boller and Wiemkin, 1986). Nonetheless,

one should expect the processes which drive the biosynthesis of these molecules to share those features which are common to known eukaryotic and prokaryotic targetting strategies, which include the presence of an amino terminal leader sequence for transport across the membrane of the endoplasmic reticulum (Saier et al. 1989; von Heijne, 1988). Finally, as a correllary to any understanding of the targetting/localization of these proteins is a demonstration of the function that these proteins serve for the plants which synthesize them. Insight into this question has been dominated by the ability of these proteins to serve as effective inhibitors of viral propagation by causing cell death. As discussed earlier, the data are inconclusive as to the ability of these proteins to inhibit their own ribosomes, a necessary prerequisite to function as antiviral agents. The case for the heterodimeric cytotoxic lectins (ricin, abrin, modeccin) as a defense mechanism against mammalian seed predators is no more convincing but less ambiguous, as there is no need for these proteins to be toxic to their own ribosome to be effective. The extracellular localization described for PAP-I is supportive of a role for that protein as an antiviral agent but is not conclusive evidence. Without further information documenting the localization of the summer leaf protein (PAP-II) and the seed protein (PAP-S) it is difficult to ascribe any function to these proteins, defensive or otherwise.

The pokeweed antiviral proteins, PAP-I, PAP-II and PAP-S comprise an assemblage of closely related proteins surmised to be transcribed from distinct genes, whose transcription is apparently related to different developmental stages of the plant. PAP-I is found in the early spring leaf tissue and persists into the summer at which time PAP-II levels gradually increase. PAP-S is never found in leaf tissue and neither PAP-I or PAP-II has been reported from the seed tissue. The PAP proteins have also been

argued to be immunologically distinct (not cross-reactive with antibody to PAP-I, but see Chapter 2). In addition, 2-D tryptic maps of the proteins appear different (Irvin et al., 1980), and the amino terminal sequences are unique (Bjorn et al., 1984). These points taken together argue for these proteins being unique gene products. This pattern of strict compartmentation, limited or inconclusive information on immunological cross-reactivity and distinct biophysical parameters between proteins with similar biological activity finds itself repeated in the glycoprotein lectins.

Lectins as Analogs to the RIPs

Lectins are abundant in seeds as seed storage proteins (Chrispeels, 1984; Pusztai et al., 1983), but it has been demonstrated that the vegetative tissues of plants which possess lectins may contain glycoproteins with similarities to the seed storage proteins. Some of these may be immunologically related to the seed lectin from the same species, but others may be distinct. Proteins similar to seed lectins have been demonstrated in tissues from Dolichos, Griffonia and Sophora (Family Leguminoseae) (Etzler, 1985) . Lectins whose levels of expression vary seasonally have been demonstrated in the bark tissues of Sambucus and Robinia (Nsimba-Lubaki and Peumans, 1986). Recently, a lectin related protein has been described in leaf tissue which is immunologically related to the seed lectin from soybean and accumulates with a lowered 'sink demand.' (Spilatro and Anderson,1989)

In addition, the expression of rice lectin mRNAs is developmentally and spatially regulated, each lectin transcript exhibiting a distinct pattern of temporal expression in the developing embryo. "Unlike animal systems, a possible correlation between multiple mRNA transcripts and tissue-specific

or developmentally regulated genes has not been established in plants" (Wilkins and Raikhel, 1989). These examples are potential homologs of the pokeweed antiviral proteins and may serve to guide our thinking about the expression and localization of these proteins.

Objectives of the Present Research

The research described here was undertaken to fulfill the following objectives: 1) To clarify the immunological cross- reactivity of the proteins from Phytolacca by the preparation of affinity purified antibodies against the three major forms of the protein (PAP-I, PAP-II, and PAP-S) with the goal to prepare specific antibody probes for these proteins. 2) To develop a tissue culture system for the expression of these proteins as a tool to examine biosynthetic processing and as a path to the establishment of a single cell suspension culture for physiologic studies on antiviral activity of these proteins and 3) To utilize the antibody probes prepared against these proteins in ultra-structural studies and as tools to examine the biosynthesis and structure of these proteins, from green leaf tissue and from the callus tissue. The following chapters provide the details of these researches.

CHAPTER II
PREPARATION AND CHARACTERIZATION OF CROSS-REACTIVE AND MONOSPECIFIC ANTIBODIES TO PAP-I, PAP-II AND PAP-S

Introduction

The pokeweed antiviral proteins are easily isolated from P. americana (Irvin, 1975) or from P. rigida (Preston and Ervin, 1987) by extraction of the homogenized leaf or seed tissues with buffer (sodium phosphate, 5mM, pH 6.2) or by the extraction of acetone powders (Preston, unpublished observation). When comparing their biophysical properties, these proteins appear very similar. However, the two proteins from leaf tissue, PAP-I (spring leaf) or PAP-II (summer leaf) and the seed protein, PAP-S are sufficiently different in ionic charge to elute differentially from a CM-52 cation exchange resin. This observation, coupled with their reported pI's, different molecular weights as determined by SDS polyacrylamide gel electrophoresis and the reported N-terminal sequences (Bjorn et al., 1984) indicated that these proteins were unique and different gene products. The amino terminal sequence data compiled by Houston et al. (1983) demonstrated 17/28 residues from PAP-I and PAP-S to be homologous. Subsequent data from Bjorn et al. (1984) demonstrated that PAP-II shared 10/29 residues with PAP-I and 11/27 with PAP-S. These data supported the interpretation of these proteins as different gene products. Limited serological data presented in several different reports strengthened the belief that these proteins were unique and antigenically dissimilar. The

following statements are excerpted from the literature addressing the relatedness of the PAP proteins and serve to illustrate the confusion surrounding this point.

The major distinguishing feature of these three proteins is found in their interaction with antibodies prepared against PAP (PAP-I). Upon challenge with anti-PAP antibodies, PAP-II fails to cross-react with the antibody as analysed by Ouchterlony immunodiffusion and by the failure of the antibody to neutralize the ability of PAP-II to inhibit cell-free protein synthesis (Irvin et al.,1980). In contrast, PAP-S has a partial cross reaction with anti-PAP antibodies as detected by immunodiffusion and furthermore, its protein synthesis inhibiting ability is neutralized by a five-fold excess of antibody over that required to neutralize the activity of PAP (Barbieri et al.,1982). from J.D.Irvin 1983. p 374.

There is no cross reactivity among the three proteins and their respective antibodies. from S. Ramakrishnan and L.Houston , 1984. p 201.

The greater degree of homology between pokeweed antiviral protein and pokeweed antiviral seed protein as compared to pokeweed antiviral protein II is consistent with immunological cross reactivity between pokeweed antiviral protein and pokeweed antiviral seed protein, but not pokeweed antiviral protein II (Barbieri et al.1982). from M.J.Bjorn et al.,1984. p 161.

Ouchterlony immunodiffusion experiments show that dodecandrin cross-reacts completely with antibody to pokeweed antiviral protein (PAP-I), indicating that all antigenic sites are held in common. . . . Although clearly not identical proteins, differing in 5/30 of the N-terminal amino acids. . . from M.P.Ready et al.,1984. p 316 -317.

Using a radioimmunoassay which easily detects 100 pg of ricin A chain, we found that no significant reaction occurred even at concentrations of PAP(I) greater than 1ug /assay tube. On the other hand, 50% of the radiolabelled ricin A chain was prevented from precipitating by a concentration of 5.3 ng/mL (0.8 ng/assay tube). Furthermore, antibodies directed against PAP do not inhibit the *in vitro* action of ricin A chain under the same conditions in which ricin antibodies completely block polyuridylic acid translation. Therefore, no common antigenic determinants exist between PAP and ricin A chain. In addition, we confirmed previous observations that antibodies generated against PAP and PAP-S do not cross react. from L.L.Houston et al.,1983. p 9602.

The overall picture to emerge from these data is that PAP-I (from P.americana) and dodecandrin (from P. dodecandra) are serologically

identical. PAP-S (from P. americana) shares antigenic determinants with PAP-I but not PAP-II. PAP-II is serologically unique. None of the PAP proteins cross-react with antibody to the ricin A chain.

It is difficult to reconcile these serological data with the biophysical and chemical similarities of these proteins. Given the overall amino acid homologies between the single chain ribosome-inactivating proteins and the A-chain of the heterodimeric toxins (such as ricin) (Houston et al., 1983; Bjorn et al., 1984), their proposed common evolutionary origins (Ready et al. 1988) and their similar enzymatic activities (Endo et al.,1988), it seemed reasonable that these proteins should share antigenic determinants in common, and that these determinants might relate to the active site of these proteins. We proposed to re-investigate the cross-reactivity of these proteins utilizing polyclonal antibody prepared against each of the three pokeweed antiviral proteins and to evaluate systematically their cross-reactivity by ELISA and by Western blotting techniques. The antibody was then affinity purified in order to prepare a panel of antibodies which showed a differential pattern of recognition, from cross-reactive to monospecific. The specificity of these antibodies when purified by immuno-affinity chromatography is demonstrated on crude extracts from leaf and seed tissue. The utility of these antibodies as probes for the expression of the PAP proteins in developmental studies was evaluated in limited studies which addressed the question of the developmental staging of the two leaf proteins, PAP-I and PAP-II. There is no developmental study on the synthesis of these proteins (and/or their subsequent decline) during the life cycle of the plant. Such a study would be of utility to gain an understanding of the significance of these proteins for the plant , as it might provide some insight into the pressures mediating the expression of these proteins and

would yield information pertinent to the developmentally linked expression of a set of closely related proteins. In the absence of a suitable nucleic acid probe to examine the levels of messenger RNA of a specific PAP protein, we have undertaken to address the question of developmental staging by probing crude extracts from P. rigida with monospecific antibodies directed against either PAP-I or PAP-II.

In addition , the ability of cross-reacting antibody to PAP-I to neutralize effectively the in vitro inhibition of a cell free translation system by the PAP proteins was evaluated.

Materials and Methods

Preparation of affinity column

Affinity columns were prepared by the coupling of pokeweed antiviral protein PAP-I, II or S to Affi-gel 10 (Bio-Rad). Protein was prepared as described (Preston and Ervin, 1987). The purity of the pokeweed antiviral proteins utilized for preparation of affinity columns and for antibody production (see below) was routinely assessed by SDS-PAGE. By this criterion, these proteins were greater than 90% pure (silver stained and Coomassie stained gels detected only one major polypeptide). In addition, amino terminal sequence data was obtained for PAP-II, and the presence of a single amino terminal sequence is presumptive evidence for the purity of this preparation. Such an analysis has not been done for the PAP-I protein.

The purified PAP proteins were individually coupled to Affi-gel 10 at a ratio of 10 mg protein/5-10 mL of gel. The gel was washed with 0.1 M MOPS, pH 7.0, and unreacted sites were blocked with 1.0 M glycine ethyl ester for 4 h at room temperature with intermittent shaking. Gel prepared in this way was then washed 5 times with PBS, 1 time with PBS containing 0.5

M NaCl, 1 time with 0.1 M Glycine-HCl (pH 2.3), and then extensively washed with PBS to restore the pH. Gels were stored in PBS with 0.02% NaN_3 at 4° C.

Preparation of antibodies

Antibodies were prepared in goats by a conventional methodology. Goats were injected with a solution containing 1.0 mg/mL of PAP-I, PAP-II or PAP-S, prepared as described (Preston and Ervin, 1987) in complete Freunds adjuvant. Antigen was readministered to goats at 10 days by a similar regime except the protein was delivered in incomplete Freunds adjuvant and the goats were then bled weekly. Antibody was isolated from the goat serum by an adaptation of a procedure which utilizes n-octanoic acid followed by ammonium sulfate fractionation (Steinbuch and Audran, 1969). Serum was titrated to pH 5.0 with 3.0 M acetic acid and then n-octanoic acid was added dropwise with stirring, to a final ratio of 1 part n-octanoic acid to 20 parts of serum. The solution was stirred at room temperature for 30 min and then centrifuged at 16,000 x g for 30 min. The supernatant solution was poured off through glass wool and an equal volume of saturated ammonium sulfate was added. The solution was stirred at room temperature for 30 min and then centrifuged for 30 min at 16,000 x g. The pellet was washed with a small volume of 50% ammonium sulfate, centrifuged and resuspended in a measured volume of PBS. The resulting purified IgG preparaation was dialysed against PBS and stored at 4°C in PBS containing 0.02% NaN_3.

Fractionation of antibody on affinity columns

Antibody prepared as described was applied to the homologous affinity column (i.e., anti-PAP-I to PAP-I, etc.) and allowed to bind at 4°C for 30 min. The column was then washed extensively with PBS containing 0.1 M NaCl , then PBS containing 0.5 M NaCl, then PBS. The column was eluted with 0.1 M glycine-HCl (pH 2.3) to elute specifically bound antibody and was then washed extensively with PBS. One milliliter fractions were collected. Fractions containing acid were neutralized by the addition of a small volume (0.2 mL) of 1.0 M Tris (pH 10.95) and the absorbance of each fraction was determined at 280 nm (A_{280}) as a measure of protein. Protein (antibody) containing fractions which washed through the column were pooled and re-applied to the column in case the protein load had exceeded the binding capacity of the column. Protein which was eluted from the affinity matrix at pH 2.3 was considered to be specific antibody. These tubes were pooled and dialysed against PBS overnight at 4°C. This antibody was then applied to a heterologous column and the procedure repeated. A simplified nomenclature was adopted to designate the specific antibody pools obtained from these trials. The antibody used in the treatment is specified first, and its affinity for the column indicated is specified (as b+ for bind and b- for not bind). For example, affinity purified antibody against PAP-I which bound to the PAP-S column is designated as I b+, S b+. A flow sheet which illustrates this process is presented in Figure 2.1. All pooled fractions from a single affinity isolation were evaluated by ELISA for reactivity to the PAP proteins (see below).

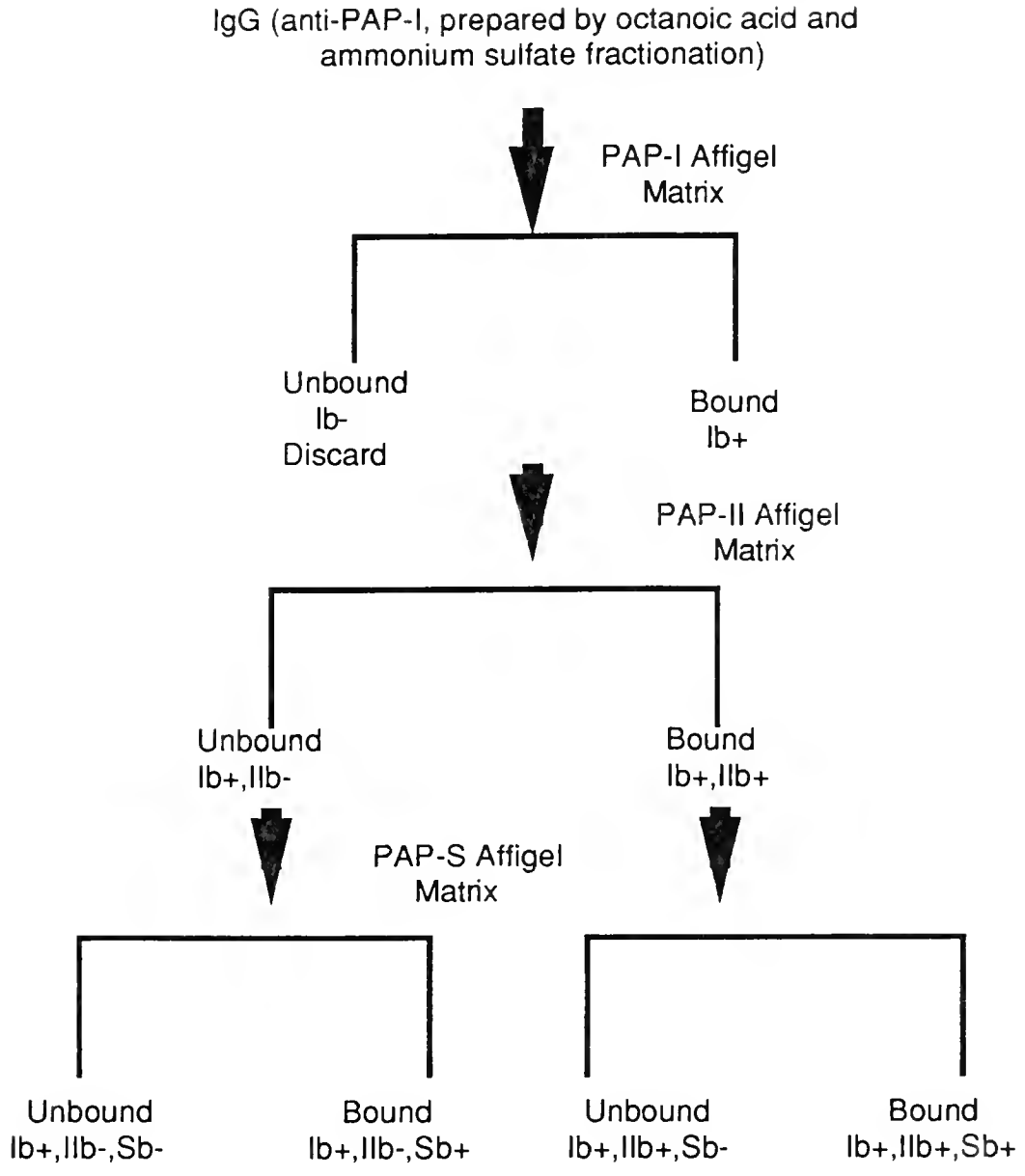


Figure 2.1. Preparation of affinity purified antibodies.

Flow-sheet of the stepwise fractionation of goat antibody to PAP-I by application to PAP-I, PAP-II and PAP-S affinity columns. Arrows are representative of the affinity column indicated. Bound (b+) and unbound (b-) fractions are indicated, preceded by the column by which they were fractionated; I for PAP-I, II for PAP-II and S for PAP-S. The shorthand notation is indicated beneath each fraction.

Preparation of plant crude extracts

Crude extracts were obtained from leaf tissue of locally (Gainesville, FL) growing wild southern pokeweed (P. rigida) Leaf tissue was collected from a population of a minimum of three different plants. Collections were made monthly, beginning in March and continuing through July (NS samples), or were collected at four day intervals (BZR samples) beginning in April and continuing through June. In addition, samples were collected from plants which sprouted in late November of 1989 and showed young leaf tissue which would be analogous to tissue from spring leaf, and from plants which showed all developmental stages including seeds at that time. Ten grams of tissue were collected and mixed with 40 mL of 5 mM Na-phosphate buffer, pH 6.5. The tissue and buffer were blended in a Waring blender at high speed for 60 s, and then filtered through four layers of cheesecloth. The resulting bright green solution was heated with intermittent stirring to 68^0 C and then cooled immediately on an ice bath to 4^0C. The resulting solution was filtered through Whatman 2V paper in the cold. The straw colored filtrate was then centrifuged at 16,000 x g for 30 min in a Sorvall RC-2B centrifuge. The supernatant solution following centrifugation was saved and assayed as described by ELISA and by SDS-PAGE and Western blot.

Enzyme linked immuno-sorbent assay (ELISA)

ELISA analysis of antibody was performed in 96 well Immulon (Dynatech) polystyrene plates. The ELISA was a direct, non-competitive ELISA in which purified antigens (i.e., PAP proteins) were plated individually as 100 uL aliquots in amounts ranging from 1 to 1000 ngs per well in 0.5 M sodium carbonate buffer (pH 9.6). Antigen was allowed to bind at room

temperature for 1 h or overnight at 4°C. The wells were washed with PBS containing 0.3% Tween-20 (PBS:Tw) three times, 100 uL/well. Antibody (100 uL/well in PBS:Tw as serial dilutions) was then added , to determine the relative titer. Antibody was bound at room temperature for 1-3 h or overnight at 4°C. The plates were incubated and washed as described. Alkaline phosphatase linked secondary antibody (rabbit anti-goat) was applied at 100 uL/well at the optimal dilution and incubated and washed as described. Color was developed from the enzymatic hydrolysis of p-nitrophenyl phosphate applied as a 1 mg/mL solution in carbonate buffer containing 0.5 mM $MgCl_2$, pH 9.1. Color development was monitored with a 405 nm filter on a Bio-Rad EIA plate reader (Model 2550).

Electrophoresis and Western blot analysis

Antigens (PAP proteins and standards) were electrophoresed at 18 mA constant current in 12% SDS-polyacrylamide gels with 4% stacking gels. The discontinuous buffer system of Laemmli (1970) was used. All buffers and gel stocks were prepared as directed in the Hoefer Catalog (Hoefer Scientific, 1988) using analytical/biochemical grade reagents. Electrophoresis was stopped when the tracking dye reached the bottom of the gel, and the gels were placed in transfer buffer (0.19 M glycine, 0.025 M Tris pH 8.3, 20% v/v methanol) for 15 min. Gels were electroblotted to nitrocellulose (Schleicher and Schuell, NC-42) for 1 h at 1 amp. The nitrocellulose was then removed from the blotting apparatus and placed into blocking buffer (PBS:Tw containing 5% (w/v) Carnation instant milk powder) for 1 h. Primary antibody containing solution was then applied in the same buffer for 1 h at room temperature with gentle shaking. The antibody solution was removed and the blots were washed 3 x 5 min each in blocking

buffer and finally enzyme linked secondary antibody was applied in blocking buffer at the appropriate dilution. After incubation for 1 h at room temperature, the blots were washed as described and then washed for 2 x 5 min each in phosphatase substrate buffer (0.1 M Tris-HCl, 0.001M $MgCl_2$, pH 8.8). Finally, substrate was applied as a solution in phosphatase substrate buffer containing 0.1 mg/mL nitro blue tetrazolium (NBT) and 0.05 mg/mL 5-bromo-4-chloro-3-indolyl phosphate (BCIP).

Gels which were to be stained with silver for protein visualization were fixed overnight at room temperature in a solution containing ethanol, acetic acid, water (4:1:5, v/v). The gels were swollen in deionized distilled water (ddw), placed into 50% aqueous methanol and then swollen in ddw prior to staining. Silver staining was performed according to the protocol of Wray et al. (1981).

Protein determination of plant crude extracts

Protein content of crude extracts was determined utilizing the BCA reagent (Pierce Chemical Co.). Bovine serum albumin (BSA) was utilized as a standard.

In vitro translation assay

The assay for the inhibitory effect of the RIPs on in vitro translation systems was performed utilizing a commercially obtained wheat germ extract (Amersham Corp.). Brome mosaic virus (Promega) was the source for mRNA for translation. Tritiated leucine (2,3,4,5 ^{3}H-L-Leucine, 115 Ci/mmole, ICN) was added to the incubation mixture and the incorporation of the radiolabel was determined by liquid scintillation counting. The procedure was as follows: 10 uL of wheat germ extract were added to a 12 x

75 mm test tube and 10 uL of the sample to be assayed were added. When assaying for the ability of antibody to neutralize the inhibitory effect of PAP proteins on in vitro translation, antibody and PAP samples were preincubated for 30 min, and then a 10 uL aliquot was added to the wheat germ extract. The samples were then incubated at room temperature for 15 min and then 10 uL of a mixture containing 19 amino acids (minus leucine) , K^+ and Mg^{2+}, brome mosaic virus RNA (1 ug) and tritiated leucine (5 uCi) were added. The mixture was incubated at room temperature for 60 min. A 100 uL aliquot of pancreatic ribonuclease (50 ug) was added and the reaction mixture was incubated a further 15 min at 37°C. The tubes were placed in an ice bath at 4°C and the protein was precipitated by the addition of 1 mL of a cold 10% (w/v) solution of trichloroacetic acid and pyrophosphate (TCAPP) in water. The acid precipitated protein was collected on a glass fiber filter (Whatman, GF/C) by vacuum filtration. The filter was washed with TCAPP (25°C), then ethanol (95%):ether in the ratio of 3:1(35°-40°C) and finally with anhydrous ether. The filter was air dried for 5 min and then placed in an oven at 60°C for 15 min. The filter was cooled to room temperature and placed in a glass scintillation vial with 5 mL of toluene based liquid scintillation cocktail and the radioactivity determined by counting in a Beckman LS spectrophotometer.

Results

Specificities and cross-reactivities of antibodies

Initial screening of goat antibody (a protein fraction enriched for IgG from goat serum prepared as described) against any of the three PAP proteins, PAP-I, II, or S by ELISA is demonstrated in Figure 2.2 (a, b and c). From these data it is clear that anti-PAP-I, anti-PAP-II or anti-PAP-S IgG

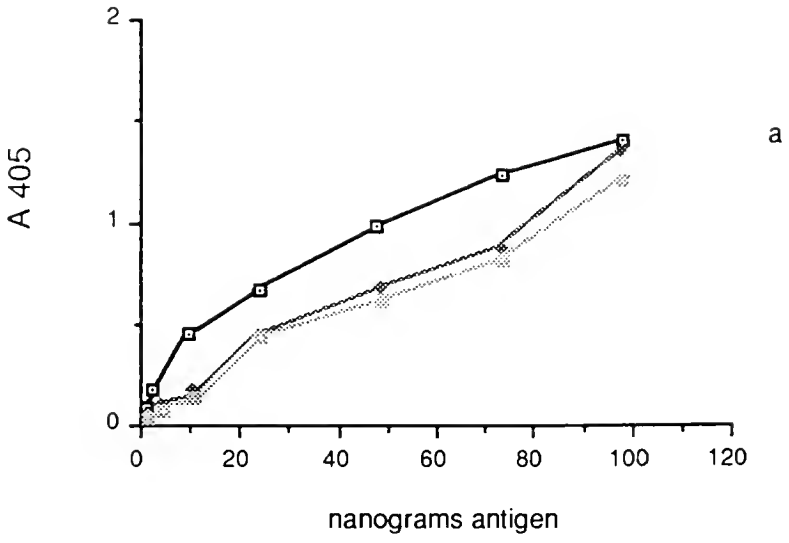
Figure 2.2. Detection by ELISA of cross reactivities between PAP-I, PAP-II and PAP-S.

PAP-I (—⊡—) PAP-II (—◈—) and PAP-S (—⬚—) were plated at amounts ranging between 3 and 100 nanograms per well in carbonate buffer as described in the text.

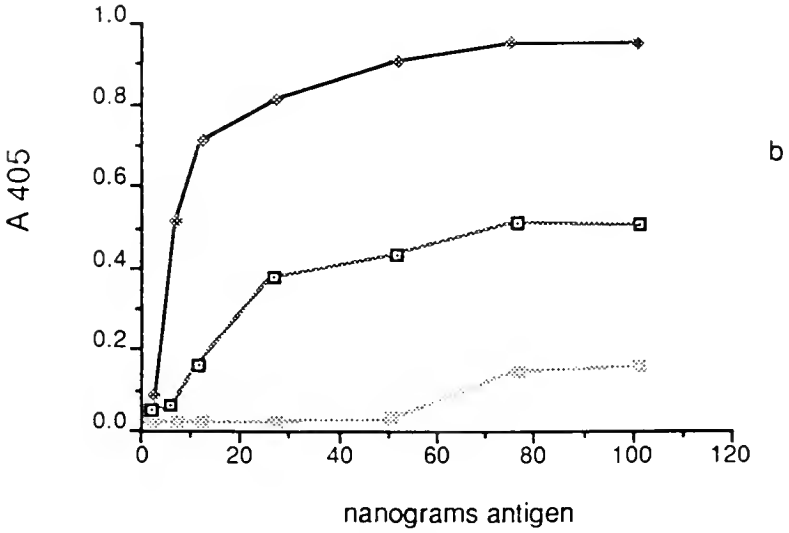
a. ELISA detection of PAP antigens with goat anti-PAP-I IgG (1/1000 dilution) prepared from octanoic acid/ammonium sulfate fractionation of serum as described in the text.

b.ELISA detection of PAP antigens with goat anti-PAP-II IgG (1/2500 dilution) prepared from octanoic acid/ammonium sulfate fractionation of serum as described in the text.

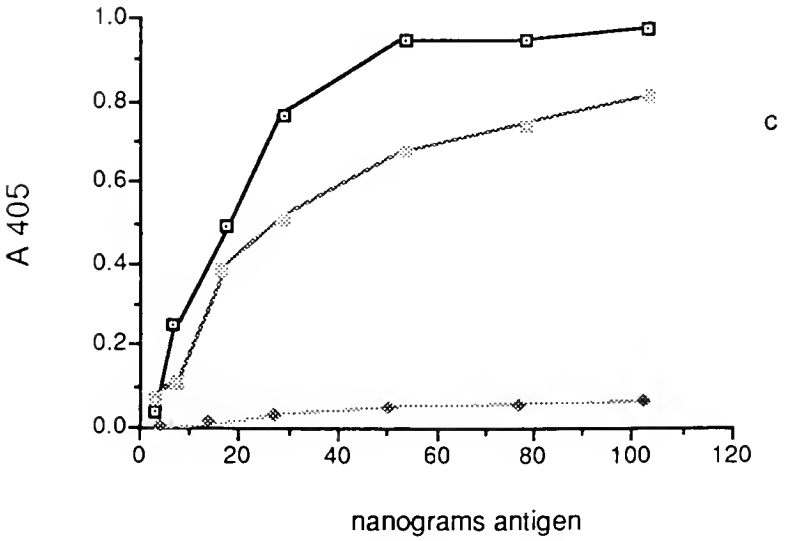
c.ELISA detection of PAP antigens with goat anti-PAP-S IgG (1/500 dilution) prepared from octanoic acid/ammonium sulfate fractionation of serum as described in the text.


A 405
2
1
0
0
20
40
60
80
100
120
nanograms antigen

a


A 405
1.0
0.8
0.6
0.4
0.2
0.0
0
20
40
60
80
100
120
nanograms antigen

b


A 405
1.0
0.8
0.6
0.4
0.2
0.0
0
20
40
60
80
100
120
nanograms antigen

c

preparations show cross-reactivity with the non-homologous PAP proteins. Anti-PAP-I (Figure 2.2 a) is cross-reactive with PAP-II and PAP-S. Anti-PAP-II (Figure 2.2 b) cross-reacts with PAP-I and to a limited extent with PAP-S. Anti-PAP-S (Figure 2.2 c) cross-reacts with PAP-I and very slightly with PAP-II. When analysed by Western blotting techniques, the cross-reactivities of these crude IgG preparations is also apparent (Figure 2.3 a, b and c). Figures 2.3 a, b and c correspond to Western blots probed with anti- PAP-I, anti-PAP-II and anti-PAP-S, respectively. In this experiment, 1.0, 0.50 and 0.25 ug of purified PAP-I (Lanes 2-4), PAP-II (Lanes 5-7) and PAP-S (Lanes 8-10) were electrophoresed and transferred to nitrocellulose as described. The Western blots were then probed with antibody as indicated. It is clear that anti-PAP-I recognizes all PAP proteins after blotting (panel a); anti-PAP-II recognizes PAP-I and PAP-II but not PAP-S (panel b), and anti-PAP-S recognizes PAP-S and PAP-I but not PAP-II (panel c). Pre-immune serum obtained from the host animals prior to injection showed no reactivity.

The preparation of monospecific and cross-reactive antibodies from each of the anti-PAP-IgG preparations proceeded by affinity chromatography (see Figure 2.1). Figure 2.4 demonstrates the elution profiles obtained from the affinity chromatography purification of anti-PAP-I by the stepwise application of this antibody preparation to a PAP-I affinity matrix (Fig. 2.4 a), a PAP-II affinity matrix (Fig. 2.4 b) and a PAP-S (Figure 2.4 c and d) affinity matrix. The antibody populations obtained after each chromatographic step were evaluated by ELISA for reactivity as a measure of the purification after each step. The ELISA data which correspond to the chromatographic separation at each step for this antibody are presented in Figure 2.5. This analysis indicated a population of the antibody was monospecific for PAP-I, and other populations which were cross-reactive. The affinity purification of

Figure 2.3. Detection of PAP proteins, PAP-I, PAP-II and PAP-S by western blotting with goat IgG against PAP-I, PAP-II or PAP-S as a measure of cross-reactivity. Antibodies were prepared as described in the text from octanoic acid/ammonium sulfate fractionation of goat serum. The lanes are the same between blots: lane 1: Molecular weight standards; lanes 2-4: PAP-I at 1.0, 0.5 and 0.25 ug, respectively; lanes 5-7: PAP-II at 1.0, 0.5 and 0.25 ug, respectively; and lanes 8-10:PAP-S at 1.0, 0.5 and 0.25 ug, respectively.

a. western blot probed with goat anti-PAP-I (1/1000 dilution).

b. western blot probed with goat anti-PAP-II (1/1000 dilution).

c. western blot probed with goat anti-PAP-S (1/1000 dilution).

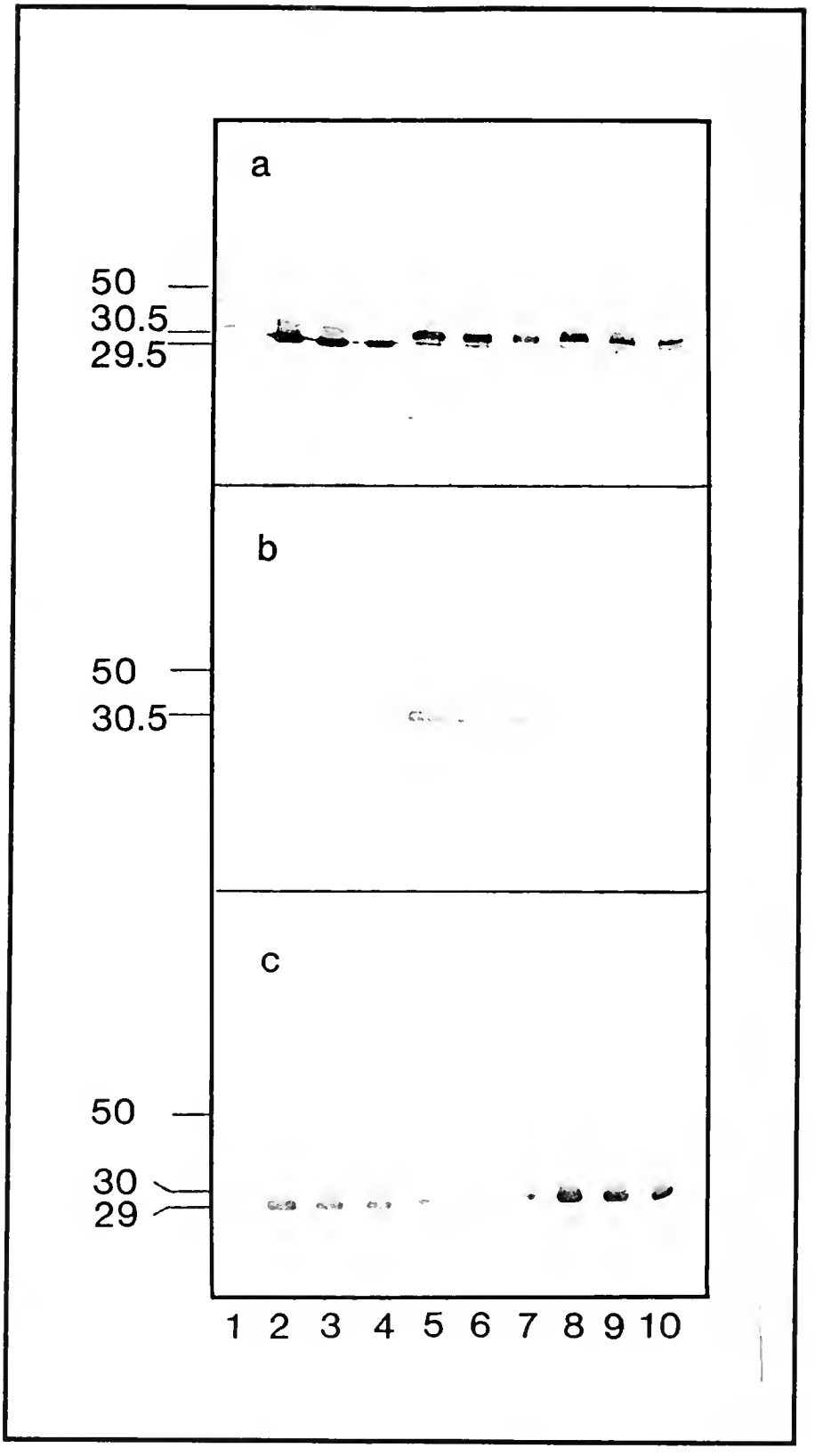

M. W.
kD
a
50
30.5
29.5
b
50
30.5
c
50
30
29
1 2 3 4 5 6 7 8 9 10

Figure 2.4. Elution profiles demonstrating the affinity purification of anti-PAP-I by the stepwise application of goat IgG antibody to PAP-I to a PAP-I column, a PAP-II column and a PAP-S column. Goat IgG was prepared as described in the text by fractionation with capryllic acid and ammonium sulfate. This IgG preparation was applied to a PAP-I affinity matrix coupled to Affi-Gel (BioRad) 10 as described, and after washing, eluted with a pH 2.3 buffer (0.1 M Glycine-HCl). Bound (b+) and not bound (b-) fractions were collected and applied to the subsequent columns.

a. anti-PAP-I applied to PAP-I affinity column

b. anti-PAP-I (b+I) applied to a PAP-II affinity column.

c. anti-PAP-I (b+I, b+II) applied to a PAP-S affinity column

d. anti-PAP-I (b+I, b-II) applied to a PAP-S affinity column

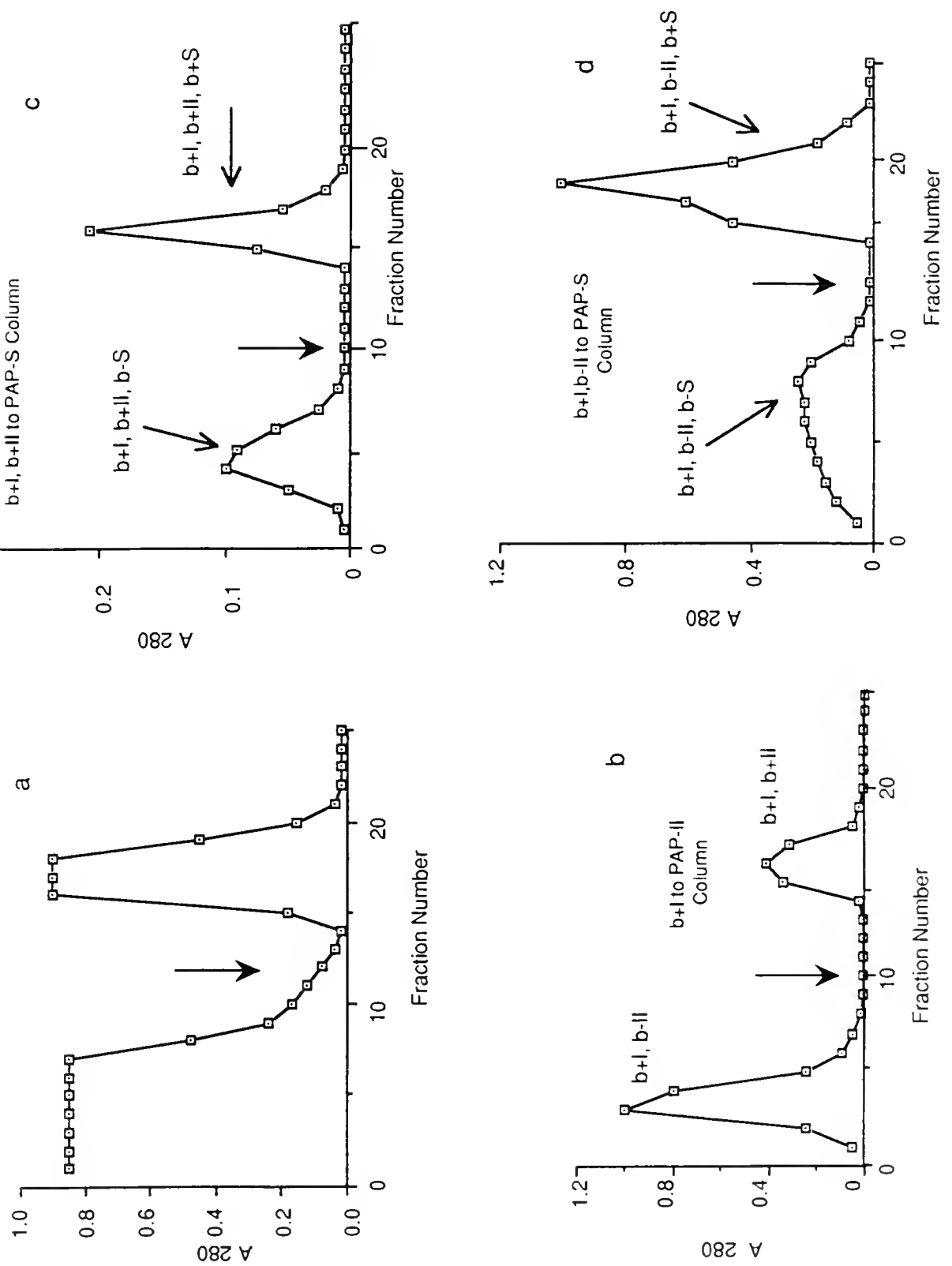
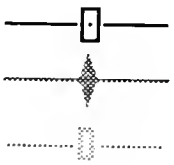

a
A 280
Fraction Number
b
b+I, b-II
b+I to PAP-II Column
b+I, b+II
c
b+I, b+II to PAP-S Column
b+I, b+II, b-S
b+I, b+II, b+S
d
b+I,b-II to PAP-S Column
b+I, b-II, b-S
b+I, b-II, b+S

Figure 2.5. ELISA analysis of antibody pools obtained by affinity purification of anti-PAP-I by chromatography on PAP-I Affigel, PAP-II Affigel and PAP-S Affigel.

Affinity chromatography was performed as described in the text, utilizing goat anti-PAP-I. The goat IgG was precipitated from serum utilizing n-octanoic acid and ammonium sulfate . Antibody was then dialyzed against TBS and applied to the affinity colums as described in Figure 2.4. The individual antibody pools obtained by this fractionation scheme were analysed by ELISA for reactivity to the heterologous antigens as described. The ELISA data correspond to the fractions described in Figure 2.4. Antigens were uniformly plated at 50 ng per well, and antibody was diluted as indicated in the figure. The symbols shown below correspond to the ELISA response with the antigen plated as described.



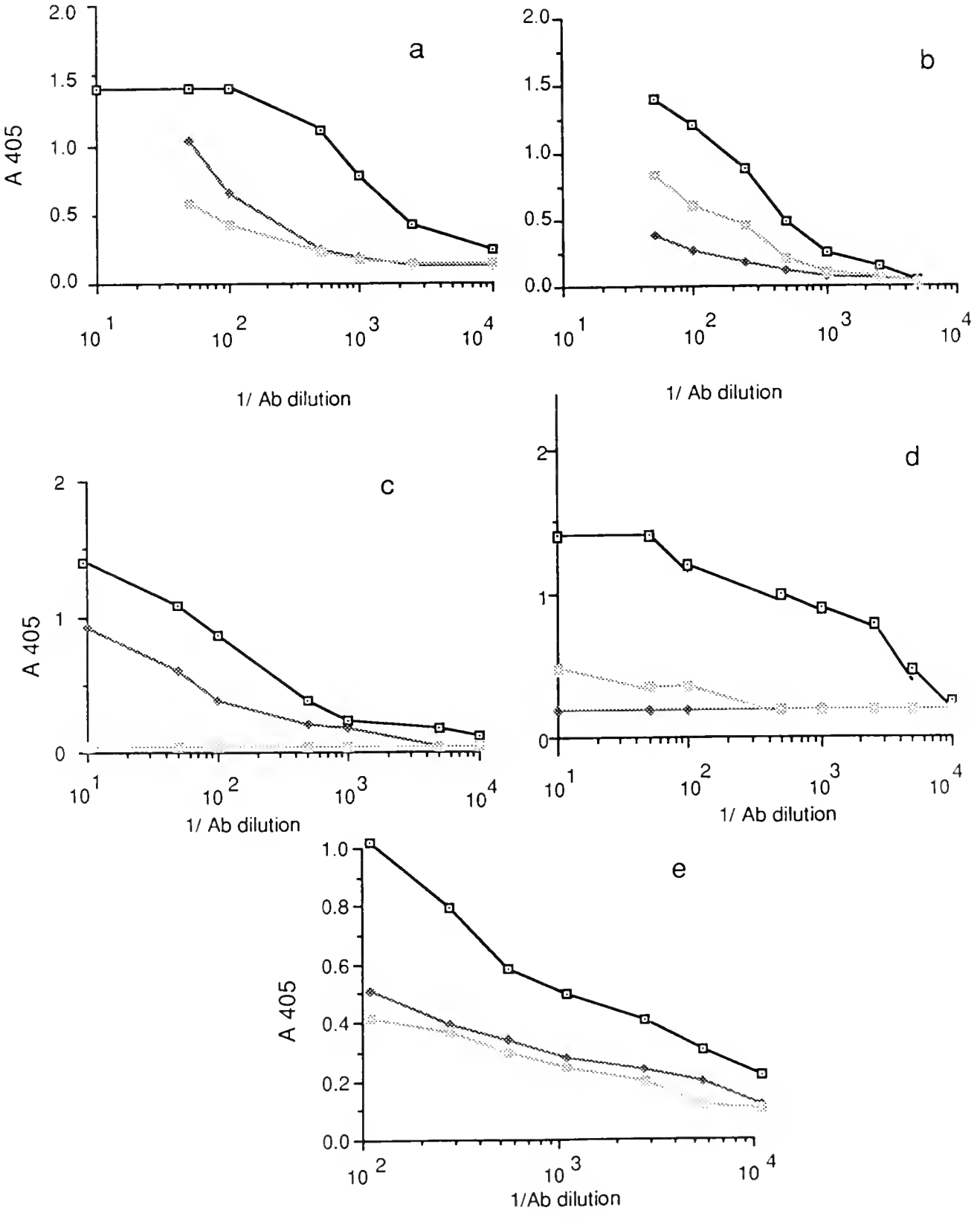
a. anti PAP-I, Ib+ (antibody dilutions as indicated)

b. anti-PAP-I, Ib+, Sb+ (antibody dilutions as indicated)

c. anti-PAP-I, Ib+,Sb-, IIb+ (antibody dilutions as indicated)

d. anti-PAP-I, Ib+, Sb-, IIb- (antibody dilutions as indicated)

e. anti-PAP-I, Ib+, Sb+, IIb+ (antibody dilutions as indicated)


a
b
c
d
e
A 405
1/ Ab dilution
1/Ab dilution

the IgG preparations for PAP-II and PAP-S proteins proceeded in similar fashion. The properties of the resultant antibodies from representative separations which figured significantly in the present research are presented in Table 2.1.

TABLE 2.1 Reactivity of affinity purified antibodies.

	REACTIVITY (dil/ug)[a]		
Antibody Preparation	PAP-I	PAP-II	PAP-S
anti-PAP-I	50	6.6	2.5
anti-PAP-I,Sb+	13.3	0	2.6
anti-PAP-I,Sb-	7.7	1.6	0
anti-PAP-I,Sb-,IIb+	5.3	2.1	0
anti-PAP-S	0.87	0	43.8
anti-PAP-S,Ib+	20	0	20
anti-PAP-S,Ib-	0	0	9.0
anti-PAP-II	0.50	6.8	0
anti-PAP-II,Ib+	0.50	0.50	0.50
anti-PAP-II,Ib-,Sb-	0	44	0

a. Calculated according to the dilution of antibody which gives an A 410 of 0.5 (on an ELISA reader after 60 min when developed against 10 ng antigen per well)divided by the amount (micrograms) of antibody used in the assay.

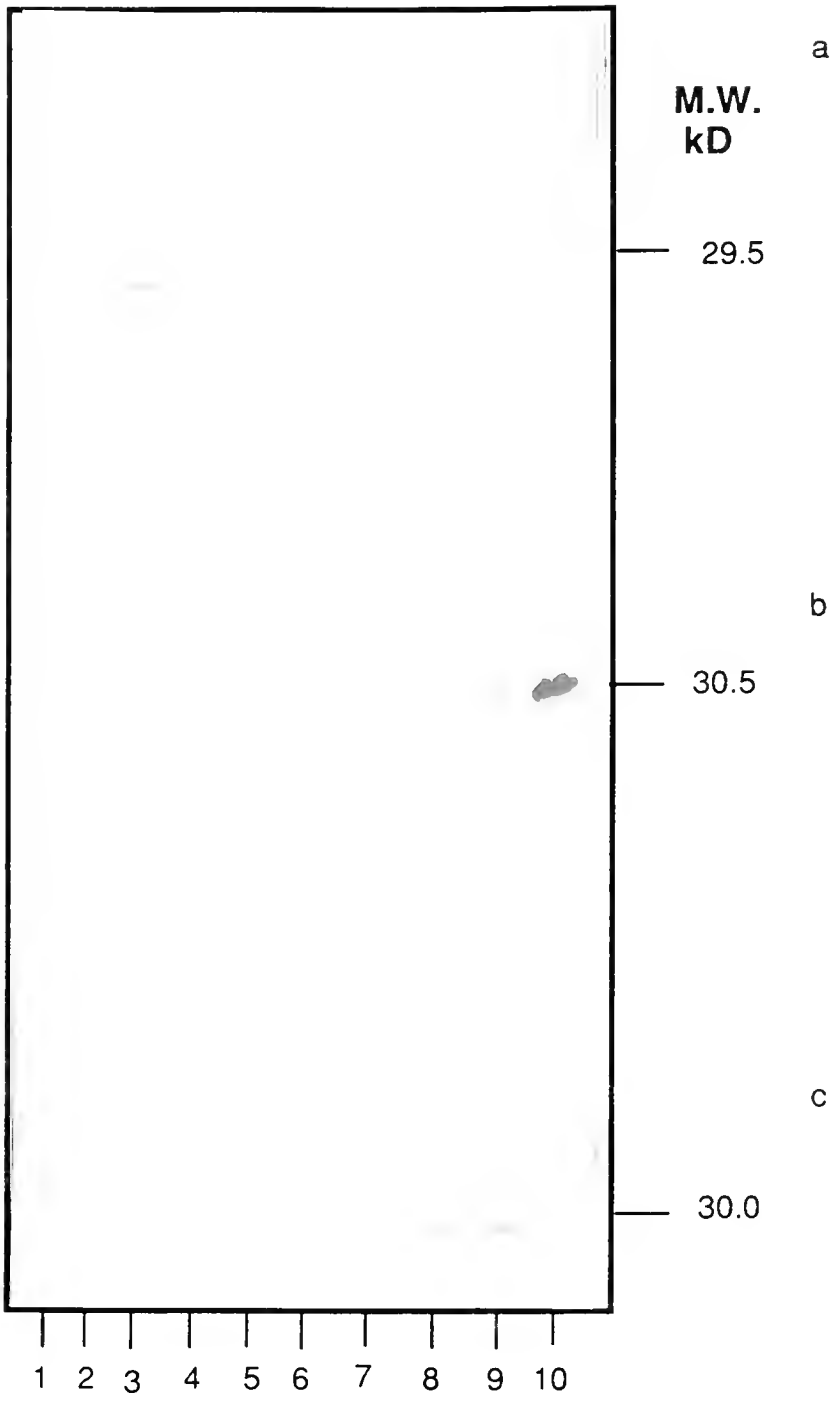
It is evident from this Table that an increase in specific reactivity of the antibodies is obtained at each chromatographic step. Of interest are the data pertaining to the preparation of the anti-PAP-II antibody, and its reactivity with PAP-S as antigen. Initially, antibody to PAP-II showed no reactivity by ELISA with PAP-S. However, following passage over the PAP-I affinity column, all antigens were equally reactive with this antibody. This

Figure 2.6. Detection of PAP proteins (PAP-I, II or S) by monospecific antibody preparations against PAP-I, PAP-II and PAP-S analysed by Western blot analysis. Crude extracts of spring leaf tissue, summer leaf tissue or seed tissue of P. rigida were prepared as described in the text and electrophoresed on 12% polyacrylamide gels as described. Western blots were prepared as described. The blots are formatted identically.
Lanes 1-3: 10 uL of a 0.1, 0.01 and 0.005 dilution of spring leaf crude extract in sample buffer, respectively; Lanes 4-6: 25 uL of a 0.25, 0.1 and 0.01 dilution of summer leaf crude extract, respectively; Lanes 7-9: 7.5 uL of a 0, 0.25 and a 0.1 dilution of seed extract, respectively. Lane 10 contained the purified antigen PAP-I, II and S at 0.5 ug each.

a. monospecific goat anti-PAP-I (1/1250 dilution)

b. monospecific gaot anti-PAP-II (1/250 dilution)

c. monospecific goat anti-PAP-S (1/250 dilution)


a
M.W.
kD
29.5
b
30.5
c
30.0
1 2 3 4 5 6 7 8 9 10

data suggests the specific enrichment of this antibody population for a small number of antibodies which are reactive with PAP-S, which reactivity is shared with PAP-I.

Ability of monospecific antibody to detect PAP antigens in crude extracts of leaf or seed tissue.

The ability of the monospecific antibodies obtained by affinity chromatography to detect the PAP antigens in crude extracts from leaf or seed tissue is demonstrated in Figure 2.6 (a, b, c). Crude leaf (spring leaf extract obtained in April, summer leaf extract obtained in July) or seed extracts (seed tissue obtained in October) were electrophoresed and Western blots prepared as described. These results indicate that the monospecific antibody pools obtained by fractionation on affinity columns show similar specificity in Western blot analyses as demonstrated by ELISA analyses (Figure 2.5 and Table 2.1). Monospecific anti-PAP-I reacted only with a single band in crude extracts of spring leaf tissue (panel a, Figure 2.6, lanes 1-3) or only with purified PAP-I (Lane 10). Monospecific anti-PAP-II reacted with some bands at the lowest dilution of crude extract from spring leaf tissue (Lane 3, panel b, Figure 2.6) but not at the position expected for PAP-II, but did react with PAP-II at the two lowest dilutions of summer leaf crude extract (Lanes 5 and 6), and with purified PAP-II (lane 10). Monospecific anti-PAP-S reacted with PAP-S in crude seed extracts at all dilutions (Lanes 7-9, panel c, Figure 2.6), and reacted weakly with PAP-I (Lane 3) at the lowest dilution analyzed. These data are consistent with the ELISA analyses and indicate that selected antibodies are specific probes of PAP-related antigens in leaf tissue of P. rigida.

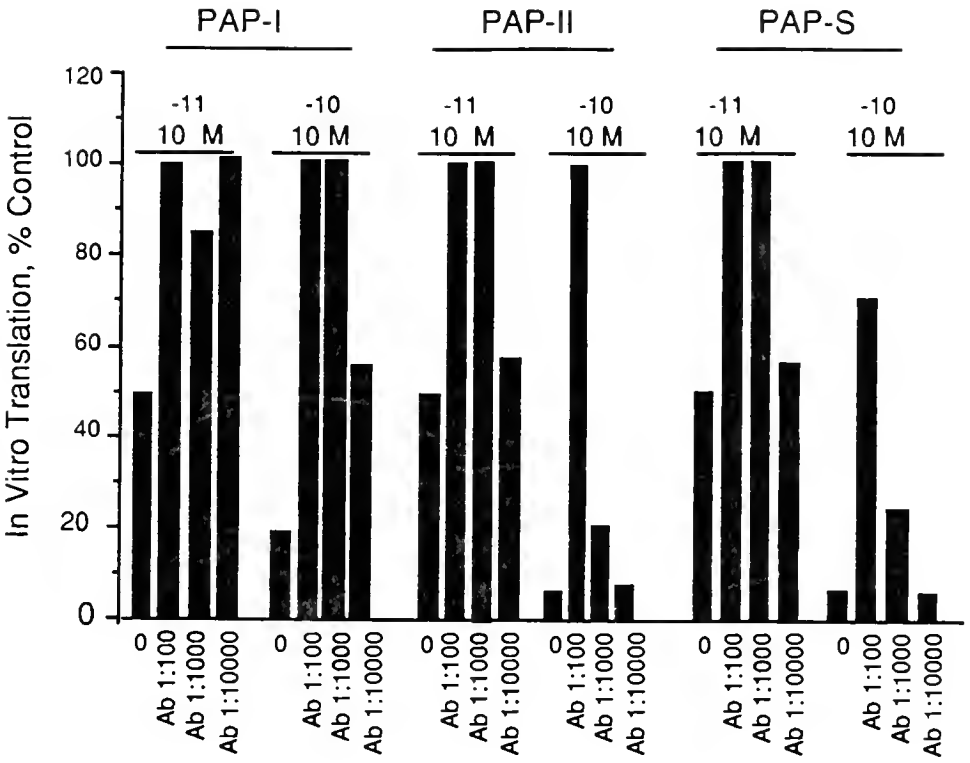


Figure 2.7. Neutralization of inhibitory activities of PAP-I, PAP-II and PAP-S by goat anti-PAP-I IgG. In vitro tranlation was determined with wheat germ extract as described in the text. Two concentrations, 10^{-11} and 10^{-10} of each form of PAP were evaluated at three dilutions of antibody (1/100, 1/1000 and 1/10000). Antibody was preincubated with the PAP protein being tested as described in the text. The figure shows for each treatment in the lane marked '0' the inhibitory effect of the PAP protein alone. The data are presented as percent of the untreated control.

Neutralization of translation inhibiting activity of PAP-I, PAP-II and PAP-S by cross-reactive anti-PAP-I.

The ability of cross-reacting antibody (prepared against PAP-I) to neutralize the inhibitory action in vitro of the PAP proteins is shown in Figure 2.7. This figure demonstrates the ability of anti-PAP-I (cross-reactive with PAP-I, II and S) at three dilutions to neutralize two different concentrations of each of the PAP proteins. The amounts of the PAP proteins were chosen so as to give approximately 50% inhibition and 90% inhibition in this assay. This corresponds to a concentration of approximately 10^{-11} M and 10^{-10} M, respectively. Antibodies were pre-incubated with the protein such that the final relative dilution of the antibody in the reaction mixture was 0.01, 0.001 and 0.0001. The data show that anti-PAP-I cross-reactive antibody effectively neutralizes the activity of the PAP proteins at low dilutions, which effect is lost with increasing dilution or in the presence of greater amounts of protein. In particular, anti-PAP-I at 0.01 and 0.001 dilution is capable of totally abrogating the ability of PAP-I, PAP-II or PAP-S at 10^{-11} M to inhibit in vitro translation. At a dilution of 0.0001, this antibody still relieves the inhibition by PAP-I by about 35% above the control, but has little effect on PAP-II or PAP-S. The ability of the cross-reactive antibody to PAP-I to neutralize the PAP-proteins is much reduced at the 10-fold higher concentration of protein. PAP-I at 10^{-10} M is still totally neutralized at the two lowest dilutions, and there is still about 35% neutralization at the highest dilution. The PAP-II and PAP-S proteins at 10^{-10} M are less susceptible to neutralization by cross-reacting antibody. PAP-II is still totally neutralized at 0.01 dilution, but this neutralizing effect of antibody is totally eliminated at the next two higher dilutions. In contrast, PAP-S is only about 40% neutralized

at 0.01 dilution of antibody, which effect is rapidly lost with increasing dilution.

Studies on the developmental staging of the PAP proteins: PAP-I and PAP-II from Phytolacca rigida

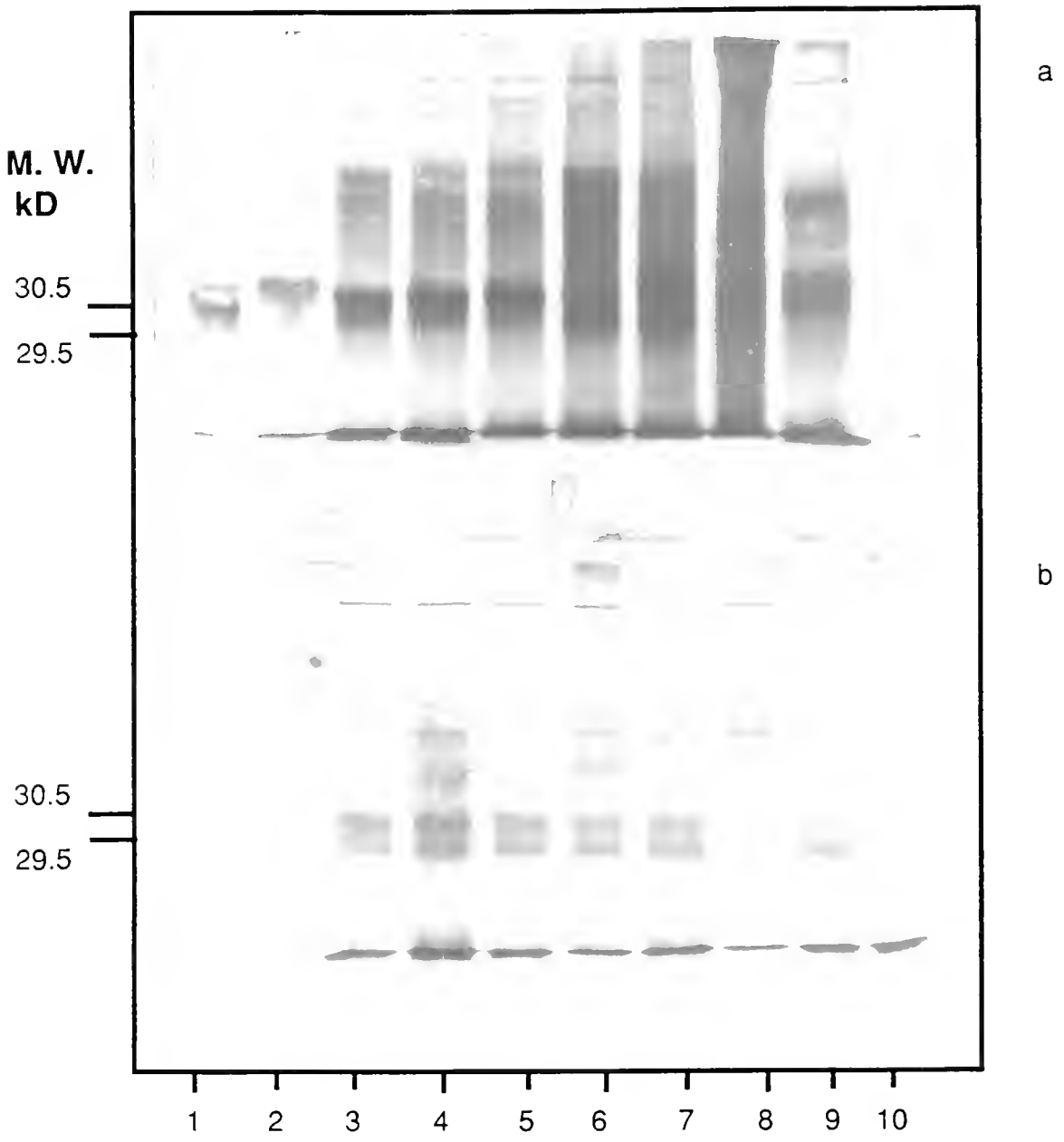
Figures 2.8 a and 2.8 b demonstrate the appearance of the crude extracts from NS samples and from BZR samples, respectively, when visualized by silver staining of SDS-PAGE gels. NS samples were collected on a monthly basis from March to August, while BZR samples represent 4 day sampling intervals from May to June. There is a gradual increase in the appearance of the PAP-II protein over the time course of the samples. PAP-I on the other hand remains relatively constant over this time period , with a slight decrease in staining density at the later time point (Lane 8, August 22). In contrast, the BZR samples, collected more frequently, do not show this same apparent increase in PAP-II (Figure 2.8 b , lanes 1-8).

The Western blot analysis of these gels is shown in Figure 2.9 and 2.10. For NS samples (Figure 2.9 a and b), there is a marked increase in immuno-reactive species detected by anti-PAP-II (panel b) over the time course of these samples while the level of anti-PAP-I reactive species (panel a) remains fairly constant. The sampling on closer time intervals (BZR samples, Figure 2.10 a and b) does not show this same clear result. Panel a, which shows anti-PAP-I reactive species does not demonstrate a clear increase in PAP-I, nor does panel b demonstrate a clear increase in PAP-II. Of interest from both of these samples (NS and BZR) is the reactivity of higher molecular weight components in crude extracts detected by anti-PAP-II antibody (Figures 2.9 and 2.10, panel b) and the reaction of this antibody with a component in crude extracts of molecular weight about 22kD which

Figure 2.8. Silver stained polycrylamide gels of crude leaf extracts from P. rigida samples collected at variuos time points during the growing season. Leaf tissue was collected at various time intervals and extracted by homogenization in buffer and heating as described in the text. Dates of collection are indicated as month/day for each sampling . Samples were centrifuged and the crude fraction obtained was mixed with an equal volume of sample buffer. The samples, 25 uL each, were electrophoresed in 12% gels as described, and stained following the procedure of Wray et al. (1981).

a. NS samples corresponding to dates March through December. Lane 1: PAP-I, 0.5 ug; Lane 2: PAP-II, 0.5 ug; Lane 3: 3/8; Lane 4: 4/10; Lane 5: 5/2; Lane 6: 6/4; Lane 7: 7/2; Lane 8: 8/22; Lane 9: 12/5.

b. BZR samples corresponding to four day interval from 4/10 to 5/12. Lane 1: PAP-I , 0.5 ug; Lane 2: PAP-II, 0.5 ug; Lane 3: 4/10; Lane 4: 4/14; Lane 5: 4/18; Lane 6: 4/22; Lane 7: 4/26; Lane 8: 5/2; Lane 9: 5/6; Lane 10: 5/10.


M. W.
kD
30.5
29.5
30.5
29.5
a
b
1
2
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5
6
7
8
9
10

Figure 2.9. Western blot analysis of the proteins detected in crude leaf extracts of P. rigida prepared from leaf tissue collected at various time intervals during the growing season. Leaf tissue was collected from locally growing specimens of P. rigida and processed by heat precipitation following blending in sodium phosphate buffer as described in the text. The blots are formatted identically. Lane 1: 3/8; Lane 2: 4/2; Lane 3: 5/2; Lane 4: 5/17; Lane 5: 6/4; Lane 6: 7/2; Lane 7: 7/21; Lane 8: 8/22; Lane 9-10: PAP-I (panel a) or PAP-II (panel b) at 0.5 and 1.0 ug, respectively. Samples were prepared for electrophoresis by boiling an equal volume of leaf extract with sample buffer for three minutes. 25 uL were electrophoresed on 12% polyacrylamide gels and Western blots were prepared as described in the text.

a. Western blot treated with monospecific goat anti-PAP-I (1/250 dilution).

b. Western blot treated with monospecific goat anti-PAP-II (1/250 dilution).

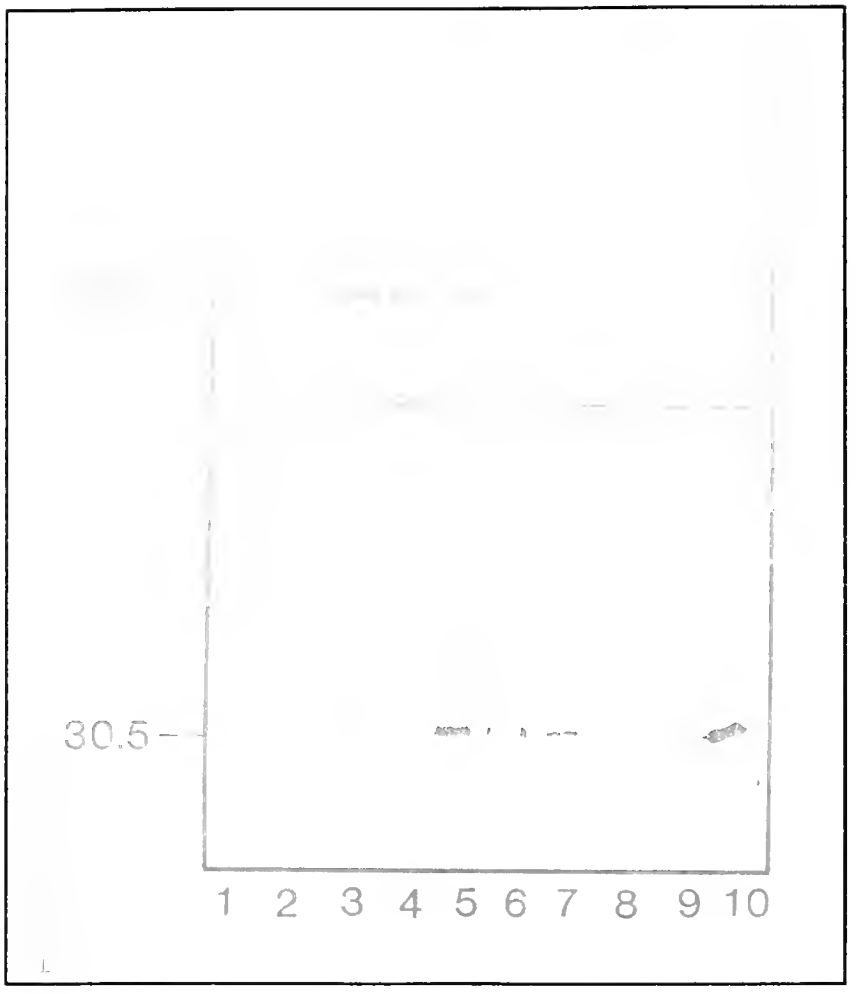
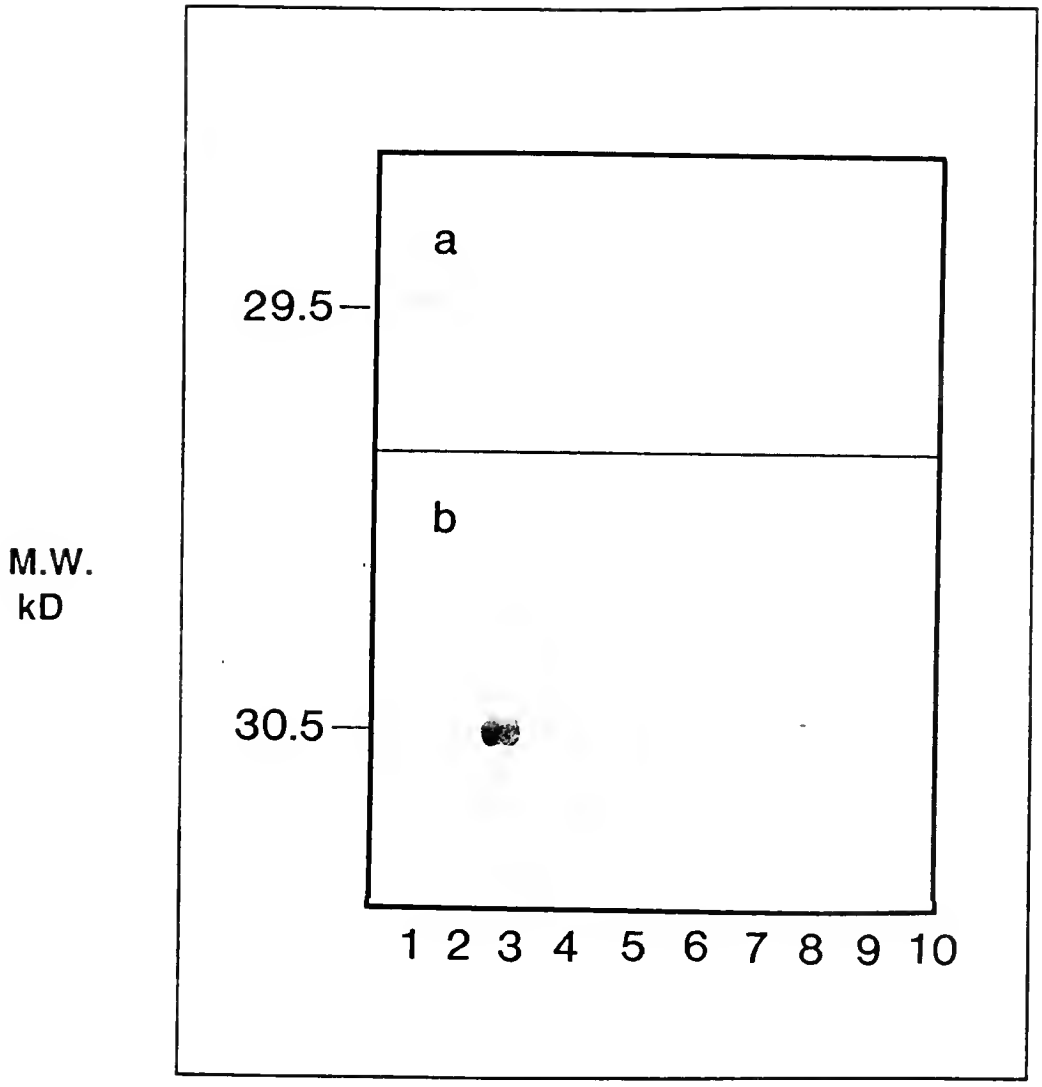

M.W.
kD
30.5
1 2 3 4 5 6 7 8 9 10

Figure 2.10. Western blot analysis of the proteins detected in crude leaf extracts of P. rigida prepared from leaf tissue collected at various time intervals during the growing season. Leaf tissue was collected from locally growing specimens of P. rigida and processed by heat precipitation following blending in sodium phosphate buffer as described in the text. The blots are formatted identically. Dates of collection are indicated as month/day. Lane 1: molecular weight standards; Lane 2: PAP-I, 1.0 ug; Lane 3: PAP-II, 1.0 ug; Lane 4: blank; Lane 5: 3/24; Lane 6: 4/12; Lane 7: 4/24; Lane 8: 5/6; Lane 9: 5/22; Lane 10: 6/18. Samples were prepared for electrophoresis by boiling an equal volume of leaf extract with sample buffer for three minutes. 25 uL were electrophoresed on 12% polyacrylamide gels and Western blots were prepared as described in the text.

a. Western blot treated with monospecific goat anti-PAP-I (1/250 dilution).

b. Western blot treated with monospecific goat anti-PAP-II (1/250 dilution).


M.W.
kD
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a
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1 2 3 4 5 6 7 8 9 10

migrates faster than PAP-I or PAP-II. These are not detected by anti-PAP-I (Figures 2.9 and 2.10, panel a)

Finally, Figure 2.11 demonstrates the results of probing young leaf tissue from plants which emerged in late October with anti-PAP-I or anti-PAP-II. Also included in this Figure are samples from the NS or BZR series (presented above) to serve as controls. For example, PAP-I is present in all leaf extracts from NS or BZR samples (Figures 2.9 and 2.10 a), while PAP-II is absent from the earliest time points in the NS series (lane 1, Figure 2.9, panel b.) Two time points were chosen from these samples as 'controls'. Figure 2.11, corresponds to a Western blot probed with monospecific anti-PAP-I (panel a) and monospecific anti-PAP-II (panel b). It is clear from this figure that PAP-I and PAP-II occur simultaneously in this young leaf tissue (see lanes 5 and 6, Figure 2.11 a and b), whereas in control lanes (lanes 1 and 3) PAP-II is not detectable in the earliest spring leaf tissue.

ELISA analyses corresponding to these data are presented in Figure 2.12. ELISA analysis was performed as described on a 100 uL aliquot of a 0.02 dilution of crude extract, and the data were corrected for protein concentration (O.D.405/ug protein assayed). Inspection of the data reveals variability in the ELISA reaction between time points within a collection. However, the overall trends inferred from the Western blots presented previously (Figures 2.9 and 2.12) are supported by these data.

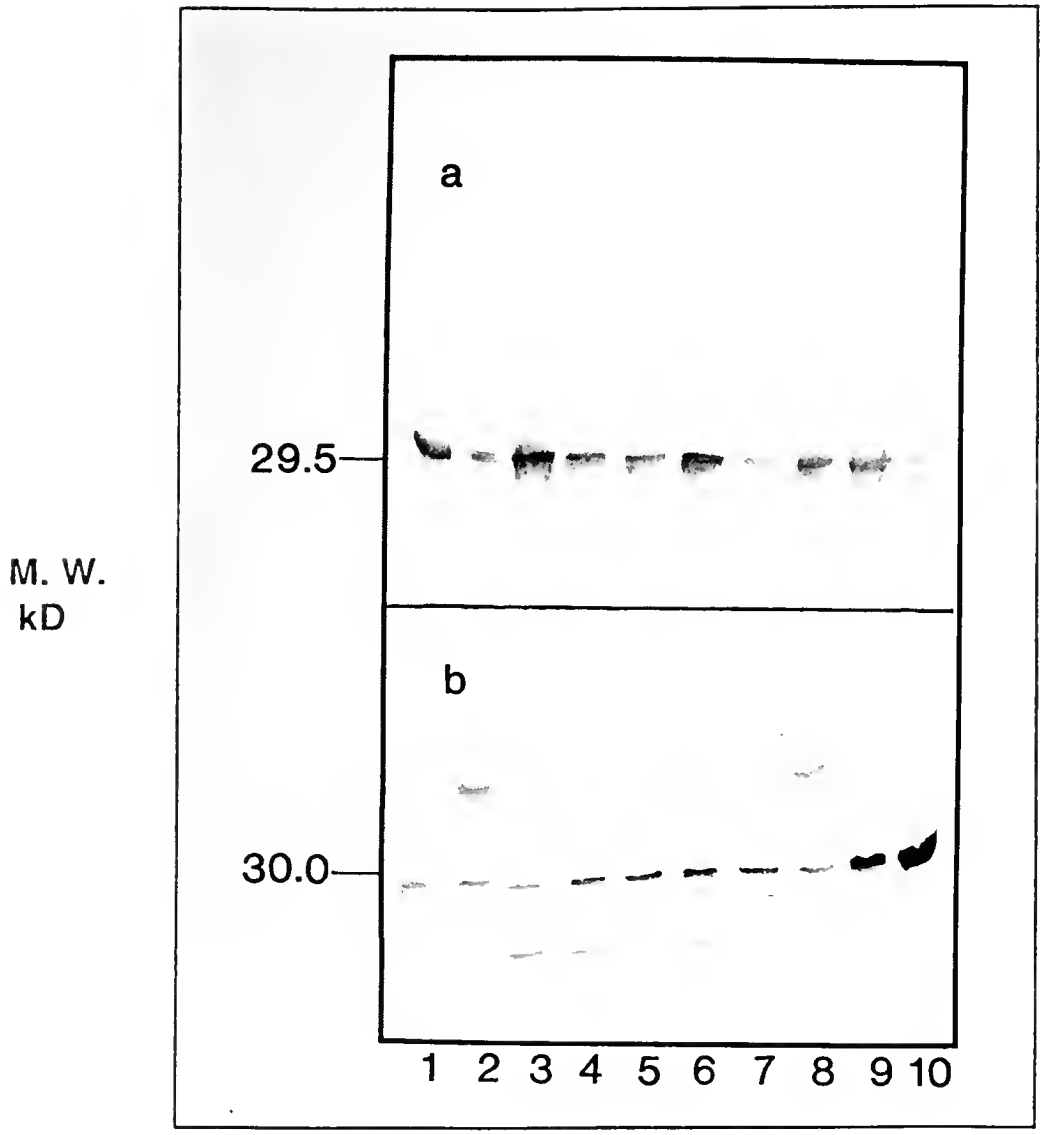
Discussion

As discussed previously, the cross-reactivity of the PAP antigens towards immune sera against one of the PAP forms (PAP-I) was not well defined. Utilizing the double diffusion technique in limited studies, it was determined that the PAP antigens were not cross-reactive (Irvin, 1975). The experiments described here were designed to address the question of the

Figure 2.11. Western blot analysis of the proteins detected in crude leaf extracts of P. rigida prepared from leaf tissue collected at various time intervals during the growing season. In particular, samples were collected from plants which showed young(newly emergent) leaf tissue in late October, 1989. Tissue was collected from locally growing specimens of P. rigida and processed by heat precipitation following blending in sodium phosphate buffer as described in the text. The blots are formatted identically.
Lane 1: 3/8/87 (NS); Lane 2: 3/24/89 (BZR), ; Lane 3: 8/22/87 (NS); Lane 4: 5/14/89 (BZR); Lane 5: young leaf tissue 10/27/89; Lane 6: young leaf tissue, second sample 10/27/89; Lane 7: leaf tissue from plant showing all developmental stages on 10/27/89; Lane 8: leaf tissue from plant showing dry berries only 10/27/89; Lanes 9-10: PAP-I (panel a) or PAP-II (panel b) at 1 or 0.5 ug respectively. Samples were prepared by boiling an equal volume of leaf extract with sample buffer for three minutes. 25 uL were electrophoresed on 12% polyacrylamide gels and Western blots were prepared as described in the text.

a. Western blot treated with monospecific goat anti-PAP-I (1/250 dilution).

b. Western blot treated with monospecific goat anti-PAP-II (1/250 dilution).


a
b
29.5
30.0
M. W.
kD
1 2 3 4 5 6 7 8 9 10

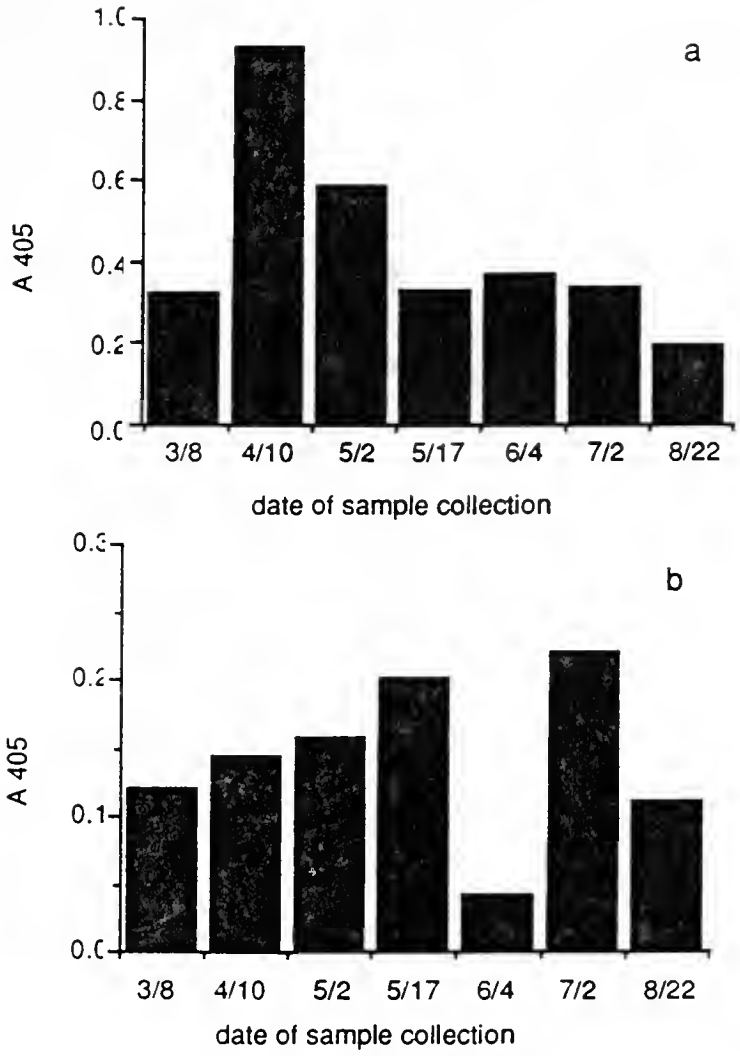
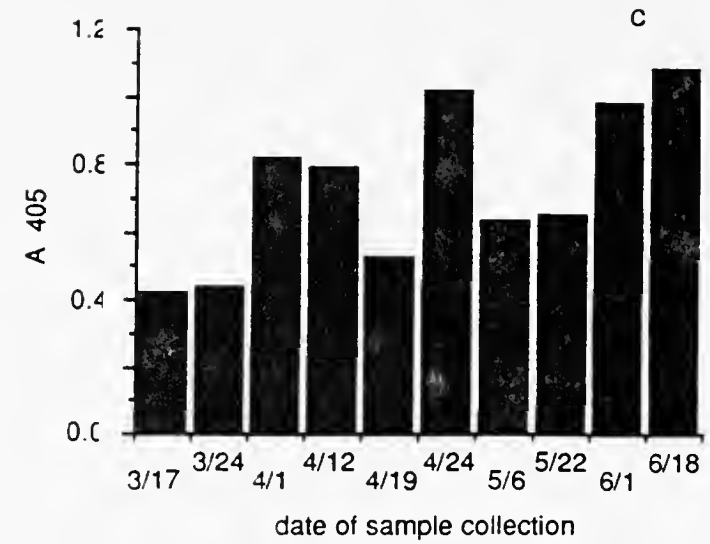


Figure 2.12. ELISA reactivity of crude extracts of leaf tissue with monospecific anti-PAP-I and monospecific anti-PAP-II.

ELISA analysis of crude extracts from leaf tissue was performed as described in the text. 100 uL aliquots of 0.02 dilutions of crude extract obtained from heat precipitation of ground leaf tissue was plated in carbonate buffer, pH 9.6. Antibody was supplied as 1: 100 fold dilutions in PBS:Tw, and secondary antibody was alkaline phosphatase conjugated. NS samples (a and b) and BZR samples (c and d) correspond to the same fractions assayed by western blotting (Figures 2.9 and 2.10) and in the SDS-PAGE of Figure 2.8 a and b, respectively.
a. NS samples probed with anti-PAP-I (1:100 dilution)
b. NS samples probed with anti-PAP-II (1:100 dilution)
c. BZR samples probed with anti-PAP-I (1:100 dilution)
d. BZR samples probed with anti-PAP-II (1:100 dilution)



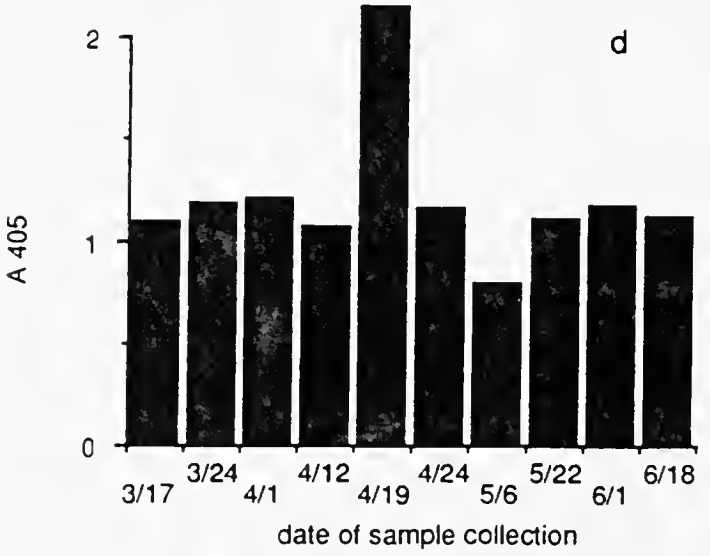


Figure 2.12 (continued)

cross-reactivity of the PAP antigens and to prepare monospecific antibodies as probes for the expression of a given PAP antigen in leaf, seed or callus tissue. In addition, it was envisioned that these probes would serve as useful tools for isolating PAP related proteins (perhaps of developmental significance) and would be of utility in immunocytochemical studies. It is clear from the data presented above that both cross-reactive and monospecific antibodies may be isolated from immune goat sera. While the argument may be made that these animals are omnivores and may have been exposed to pokeweed as a forage food, thus provoking the cross-reactivity observed, two observations mitigate against this possibility. 1) Pre-immune sera show no reactivity at the dilutions where the immune sera are reactive, and 2) PAP-S as antigen shows very limited cross-reactivity with PAP-II, and PAP-II as antigen shows no reactivity with PAP-S. This result would not be expected if the goats were feeding freely on pokeweed and supports previous serological observations obtained with studies utilizing rabbit antibody. This same pattern of reactivity has been observed in our laboratory utilizing antibody prepared in mouse (Preston and Ervin, 1987), chickens and rabbits (Preston, unpublished observations). The cross-reactivity observed may depend upon the sensitivity of the assay system utilized in the present study. Typically, 10 ng are easily detected by the current ELISA system. If one measures reactivity by ELISA and then standardizes the observed response (color development) by factoring in the amount of antibody protein in the reaction, one can arrive at a measure of cross-reactivity. Such an analysis was performed by Preston and Ervin (1987). They were able to demonstrate that anti-PAP-I had a reactivity of 1 with PAP-I, 0.46 with PAP-II and 0.11 with PAP-S. Ricin A chain was of limited reactivity (0.04). The data presented in Table 2.1 are in agreement

with those data reported previously. In addition, the ability of cross-reacting antibodies to neutralize the enzymatic activity of the PAP proteins (Figure 2.7) argues for the presence of shared epitopes. Those epitopes which are shared may be near the active site, although an influence on activity due to conformational changes in the PAP protein on binding antibody cannot be ruled out. Antibody alone at the lowest dilution used here had no effect on in vitro translation when supplied to the reaction mixture.

From the data presented here, it is clear that the PAP proteins may be effectively immobilized on Affi-gel. This result would be anticipated due to the highly basic pI values reported for these proteins. In addition, coupling via iso-amide linkage does not affect the ability of the PAP proteins to bind antibody. This is crucial for the successful utility of these columns as reagents for purifying monospecific antibody.

The major points to emerge from these results may be summarized as follows.

1). Purified IgG or crude antisera show cross-reactivity. PAP-I is cross-reactive with all PAP species. PAP-II and PAP-S are negligibly cross-reactive, but each is cross- reactive with anti-PAP-I.

2). The highly basic proteins PAP-I, PAP-II and PAP-S are easily coupled to NHS-activated Sepharose and retain their antigenicity following covalent linkage to Sepharose.

3). Antibody prepared by affinity chromatography showed the expected specificity and both cross-reactive and monospecific antibody preparations could be obtained by affinity chromatography.

4). Monospecific antibody prepared by immunoaffinity chromatography recognizes the native antigen from crude extracts on Western blots. By this

criterion , PAP-S is not found in crude leaf extracts and PAP-I or PAP-II is not found in crude seed extracts.

5). Cross-reactive antibody (made against PAP-I) is able to neutralize the inhibitory activity of PAP-I, PAP-II or PAP- S in a wheat germ translation system.

PAP-I and PAP-II are closely related proteins which occur in leaf tissue of P. americana and P. rigida. PAP-I is associated with and isolated from 'spring' leaf tissue, and PAP-II is isolated from 'summer' leaf tissue. The present report deals with the immuno-detection of these proteins in crude extracts from leaf tissue utilizing monospecific antibody probes to PAP-I and PAP-II. From the data presented in Figure 2.9, it is clear that PAP-I (Figure 2.9, panel a) is the dominant protein in leaf tissue collected in March-April. PAP-II increases over the course of the sampling period (Figure 2.9, panel b), until PAP-I and PAP-II occur simultaneously in the late summer leaf tissue (Figures 2.9 and 2.10, panel b). The appearance of PAP-II in late leaf tissue has been related to an undefined (transcriptional or translational) "switch" (Lord and Roberts, 1987) in the expresssion of these two forms. It is clear that PAP-II increases, but not at the expense of PAP-I. PAP-I remains fairly constant in its expression (at least as detected by immunoblot) over the time course of the sampling period. This methodology cannot distinguish whether the increase in PAP-II is related to an increased transcriptional rate or an increase in translational activity. Neither can one eliminate the possibility of regulated protein degradation as a mechanism for controlling the relative amounts of these proteins in leaf tissue.

As a percent of total protein, the ratio of PAP-I to PAP-II does decrease, further confirming the apparent increase in PAP-II. In addition, the apppearance of higher molecular weight species uniquely detected by

monospecific anti-PAP-II is of interest. These components appear to increase in amount with respect to the sampling period (see Figures 2.9 and 2.10, panels b). Previous work in this laboratory (Ervin and Preston, 1988, and see Chapter 3) has identified the presence of putative intermediates of PAP-II in tissue cultures from P. rigida and the presence of these might not be unexpected in crude extracts from leaf tissue. Boness and Mabry (Dept. of Botany, Univ. Texas, Austin , personal communication) have obtained information on the appearance of PAP-like proteins of altered electrophoretic mobility from suspension cultures of P. dodecandra. P. dodecandra is reported to produce a protein (dodecandrin) which is homologous to PAP-I from P. americana (Ready et al., 1984), and by logical extension one that should be homologous to PAP-I from P. rigida , as well. P. dodecandra is not reported to possess a homolog of the PAP-II from these two other Phytolaccca species.

The idea has been promulgated in the literature surrounding these proteins that their association with 'spring' leaf tissue or 'summer' leaf tissue reflects a temporal sequence of biosynthetic events. However, by analysis of young leaf tissue from plants which emerged late in the year, we were able to show that PAP-I is a constitutive part of all leaf tissue, young or old (Figures 2.9, 2.10 and 2.11). PAP-II levels increase over the course of the growing season, but in young leaf tisssue from plants newly emerged late in the growing season (i.e., November), the levels of PAP-I and PAP-II as detected by immunoblotting/ELISA are about the same. (see Figures 2.11 and 2.12). The ELISA data in Figure 2.12 have implications for a sampling strategy designed to elucidate developmental expression of these proteins. When sampled on a monthly basis (Figure 2.12 a and b), trends in the expression of PAP-I (Figure 2.12 a) and PAP-II (Figure 2.12 b) are apparent.

However, when trying to define more narrowly the window of expression by sampling on more closely (four day) spaced intervals for PAP-II , no such trends are apparent. This observation suggests that the expression of PAP-II in 'summer' leaf tissue is a gradually cumulative effect, and not an all-or-none event.

The antibodies prepared in this study have been shown to be useful immunologic probes for the presence of PAP related antigens in tissue extracts and are thus candidates for extended studies utilizing immunogold techniques or in polysome isolations.

CHAPTER III
TISSUE CULTURE STUDIES

Introduction

The pokeweed antiviral proteins, PAP-I, PAP-II and PAP-S are ribosome-inactivating-proteins (RIPs) which have been isolated from the spring leaves, summer leaves and seeds respectively, of P. americana (Irvin,1983; Stirpe and Barbieri,1986). These three forms of the PAP protein are all basic proteins with pI values of 8.1, 8.5, and 8.3 and molecular weight values of 29.5 kD, 30.0 kD and 31.0 kD for the PAP-I, PAP-S and PAP-II proteins, respectively. When isolated from green plant tissue, no carbohydrate is detectable on any of the mature PAP forms. Similar, if not identical forms have been isolated and purified from the southern pokeweed, P. rigida (Preston and Ervin, 1987).

Each PAP protein has nearly the same inhibitory activity toward in vitro translation systems (Irvin, 1983; Preston and Ervin,1987). The pokeweed antiviral proteins are typical of other RIPs in that their inhibitory activity is the result of an N-glycosidase, depurinating a specific site in the 28S rRNA (Endo et al.,1988; Stirpe et al., 1988). The occurrence of three forms of this protein, apparently independently transcribed from different genes (Ready et al., 1984), has provoked my investigations into the developmental biosynthesis of these proteins. I have established callus cultures from the spring leaves of P. rigida and demonstrate the ability of this callus tissue to synthesize two forms of the PAP protein, PAP-I and PAP-II, which are detected by affinity purified antibodies prepared against the proteins

isolated from green leaf tissue. In addition we present evidence for glycosylated forms of the proteins reactive with anti-PAP-II.

Immunocytochemical studies were employed to localize proteins bearing PAP-I or PAP-II epitopes in the callus tissue. The localization of the anti-PAP-I cross reactive material and anti-PAP-II cross reactive material (crm) from the callus tissue was examined.

Structural comparisons of the PAP-like proteins from callus tissue with native leaf proteins were performed by cyanogen bromide mediated cleavage to establish the identity of the tissue culture proteins with the leaf proteins.

Materials and Methods

Chemicals, reagents and media

All chemicals for the preparation of media were ACS certified or biochemical grade. CM-52 cation exchange resin was from Whatman, the ion retardation resin AG 11X8 was from BioRad and Sephadex G-75 gel filtration resin was from Pharmacia. All commercial antibodies and reagents for ELISA and EITB were from Sigma. PAP-I, PAP-II and PAP-S standard proteins were isolated and purified from locally growing P. rigida using minor modifications of published procedures (Irvin, 1975; Barbieri et al.,1982; Preston and Ervin, 1987). All proteins were prepared by cation exchange chromatography on CM-52 resin followed by gel filtration on Sephadex G-75 in 0.1M NH_4HCO_3. Each protein after purification gave a single band on SDS-PAGE when detected by Coomassie blue with apparent molecular weights of 29.0, 30.0, and 31.0 kD for PAP-I, PAP-S and PAP-II, respectively.

Preparation of monospecific antibodies

Monospecific and cross-reactive antibodies directed against PAP-I, PAP-II and PAP-S were generated in goats and purified by affinity chromatography as described previously (Chapter 2).

Preparation of antibody to tissue culture proteins

Proteins were purified from callus tissue as described below. Proteins bearing PAP-II epitopes were isolated as 44 kD and 34 kD fractions. These were prepared as 0.1 mg/mL solutions in PBS and injected into chickens. Antigen solutions were injected subcutaneously at 0 and at 14 days into the wing (0.7 mL) and into the foot pad (0.3 mL). Antibodies against both tissue culture proteins were obtained from immunized chicken egg yolks by an adaptation of the procedure initially described by Jensenius et al. (1981). Briefly, yolks were isolated from the surrounding fluid and washed gently with 50 mL of PBS. The washed yolk was disrupted at room temperature by mixing (magnetic stirrer) in five times the volume of ddw. The pH of the resulting solution was adjusted by the addition of 1N NaOH to 7.0 after stirring for 15 min. This solution was frozen and then thawed to room temperature. After thawing the solution was centrifuged for 30 min at 10,000 rpm in a GSA type rotor in a 150 ml polycarbonate tube. The resulting supernatant fluid was assayed directly, following filtration through three layers of cellulose tissue (KIMWIPE), by ELISA. Screening of egg yolks by this rapid freeze-thaw method allowed the identification of eggs which showed high titers of these antibodies. These eggs were pooled and processed by the method of Polson et al. (1985) which involves precipitation of the immunoglobulin fraction from egg yolk with polyethylene glycol.

Electrophoresis

Analysis of specific fractions by SDS-PAGE was performed in a Hoefer vertical slab gel apparatus with 4% stacking gel and 12% running (separating) gel as described by Laemmli (1970). Electrophoresis was performed at a constant 150 V for 1 h and the voltage was then increased to 250 V for 2.5 h. Gels were removed and stained with 0.1% Coomassie blue for 1 h and destained in ethanol (95 %): acetic acid: water (60:10:30) until the background was clear. Alternatively gels were stained with ammoniacal silver according to the method of Wray et al. (1981). Gels which were to be utilized in western blotting experiments were placed into transfer buffer (0.192 M glycine, 0.025 M Tris, pH 8.3, 20 % v/v methanol) for 30 min prior to transfer.

Gels which were utilized for the analysis of low molecular weight peptides generated from the cyanogen bromide hydrolysis of the PAP and PAP-like proteins from callus were prepared according to the methodology of Giulian and Graham (1985) and visualized with the silver staining methodology described by Giulian et al. (1983). As it was difficult to obtain uniform results with the electrophoretic transfer from these gels, gels to be utilized in the western blot analyses of these low molecular weight peptides were prepared according to the methodology described by Christy et al. (1989). This method involves the addition of 0.1 M NaOAc to the anode buffer during electrophoresis and is otherwise identical to SDS-PAGE as described by Laemmli (1970).

Western blot analysis (EITB)

Gels which had been equilibrated in transfer buffer were assembled in cassettes and then transferred for 1 h at 1 amp (constant current) in a Hoefer transblot apparatus. Nitrocellulose membrane was from Schleicher and

Schuell (NC-42). After transfer, membranes were incubated in PBS:Tw:BSA (O.1 %) for 1 h, then in antibody containing solution at the appropriate dilution for 1 h. Membranes were washed 4 x 15 min in PBS:Tw:BSA and then incubated in rabbit anti-goat IgG conjugated to peroxidase. Color was developed from the oxidation of benzamidine-HCl. Alternatively, the blots were developed by exposure to secondary antibody conjugated to alkaline phosphatase, in which case the color was developed from NTB (nitrotetrazolium blue) and BCIP (5-bromo-4-chloro-3-indolyl-phosphate).

Lectin binding to Western blots

The binding of the lectin Concanavalin A (ConA) to proteins following blotting to nitrocellulose was performed according to the following procedure. Proteins on SDS-PAGE gels were electrophoretically transferred to nitrocellulose membranes as described. After transfer, the blots were incubated in PBS containing gelatin (3 %), 1 mM Ca^{2+}, 1 mM Mg^{2+}, 0.1 mM Mn^{2+} ($PBSG^{+}$) for 1 h. The blots were then placed in a solution containing ConA (10 ug/mL) in $PBSG^{+}$ or with ConA plus 0.1 M alpha methyl-D-mannoside for 1 h at room temperature. Following exposure to the ConA, the blots were washed 3 X15 min in $PBSG^{+}$, and then exposed to goat anti-ConA at the optimal dilution. The blots were incubated for 1 h at room temperature, washed as described and incubated with rabbit anti-goat IgG conjugate to alkaline phosphatase at the optimal dilution for 1 h. After washing, the blot was developed with tetrazolium blue with BCIP as the substrate. Ovalbumin and ricin A chain (ICN) were included on the gels as molecular weight markers for glycoproteins.

Enzyme-linked-immuno-sorbent assay (ELISA)

ELISA analysis was performed on dilutions of samples plated as 100 uL aliquots on Immulon polystyrene plates. The ELISA was a direct, non-

competitive ELISA which allowed for the rapid screening of chromatographic fractions. (see Chapter 2)

Carbohydrate

Carbohydrate was determined by the phenol/sulfuric acid method of Dubois et al. (1956) with the following modifications. Five hundred uL of sample were mixed with 25 uL of 80% (v/v) phenol in water in a 13 x 100 mm test tube and 1.25 mL of concentrated sulfuric acid was rapidly added . Glucose was used as standard and all data are presented as gram equivalents of anhydrohexose. Descending paper chromatography of acid hydrolysates was utilized to determine the major carbohydrates present. Hydrolysis of isolated glycoprotein was performed in 3.0 N HCl for 3 h at 110° C. The hydrolysate was lyophilized repeatedly from distilled water and then resolubilized in a minimal volume of distilled water. The material was passed through an ion retardation column and the neutral sugar fraction was collected, pooled and concentrated by rotoevaporation under vacuum at 40° C. Aliquots were spotted on Whatman No.1 chromatography paper and chromatographed in pyridine: butanol: water (6: 1: 3) for 24 h. Carbohydrate was detected by staining the chromatogram with ammoniacal silver nitrate (Trevelyan et al., 1950).

Glucose was quantified by a coupled enzyme assay utilizing hexokinase and glucose-6-phosphate dehydrogenase to catalyze the conversion of glucose to glucose-6-phosphate to 6-phosphogluconate. The reduction of NADP to NADPH was monitored by measuring the absorbance at 340 nm. With NADP in excess, the reaction was followed to completion on a recording spectrophotometer. The stoichiometric conversion of glucose to 6-phosphogluconate and NADP to NADPH was quantified using a molar

absorptivity of 6.22 $mM^{-1}cm^{-1}$ for NADPH (CRC Handbook of Biochemistry, 1970).

Peptide sequencing

Amino terminal sequence of peptides was performed by automated Edman degradation utilizing a sequencer (Applied Biosystems) in the Protein Core Facility , Department of Biochemistry and Molecular Biology, University of Florida. Protein prepared as described below was dialyzed extensively against distilled water to remove trace amounts of ammonium bicarbonate and lyophilized. Alternatively, protein was blotted directly to PVDF membrane (Millipore) following SDS-PAGE and sequenced directly from the blot (Matsudaira,1987).

Protein synthesis inhibition

The in vitro translation system derived from wheat germ extract (Amersham) was assayed by the incorporation of 3H-leucine into acid insoluble product as described previously. The ability of isolated proteins to inhibit this activity was assayed by preincubating the extract with the protein sample of interest, and then assaying for activity as described. (see Chapter 2)

CNBr cleavage of proteins

PAP proteins from leaf tissue and callus were prepared as previously described. Each protein (1 mg) was dialysed against ddw overnight to remove salts and then lyophilized. The lyophilized powders were solubilized in a 0.50 mL volume of 70% (v/v) solution of formic acid. A measured volume of CNBr in 70% (v/v) formic acid was added so as to yield a 100-fold excess of CNBr over methionine. The reaction mixture (final volume < 1.0 mL) was placed in an hydrolysis tube, flushed with nitrogen and allowed to stand overnight at room temperature. The reaction was

stopped by the addition of ten volumes of ice cold ddw and lyophilized. The lyophilized product was resolubilized in ddw and lyophilized. This process was repeated three times.

Callus establishment.

Fresh leaves were collected from locally growing specimens of P. rigida. Leaf tissue was washed well in sterile phosphate buffered saline (PBS), rinsed three times in sterile water and briefly immersed in a 3% solution of hypochlorite. After several rinses in sterile water , leaf segments were placed onto a modified Murashige-Skoog (M-S) medium (pH 5.7) which contained in addition to the M-S salts (Smith, 1986), 2 g/L casein hydrolysate, 30 g/L sucrose, 0.1 g/L myo-inositol, 2,4-dichloro phenoxy acetic acid (4.5 uM) , kinetin (0.9 uM) and the vitamins nicotinic acid (50 ug/L), thiamine-HCl (40 ug/L) and pyridoxine (50 ug/L). Leaf explants were maintained in the dark at room temperature. White callus produced from the edges of the cut tissue was excised and replated onto fresh medium. A firm, viable callus was established after two passages and has since been maintained on this medium, transferred every fourteen days. Several of the calli obtained produced a red-pigmented tissue. These were maintained as a separate line from the white tissue. The red pigmented callus was stable over the duration of these experiments. Callus cultures of both lines are shown in Figure 3.1.

Fractionation of proteins.

Callus tissue harvested for these experiments was stored at 4°C for four days. Alternatively, tissue was stored at -20°C immediately after harvesting

a b

Figure 3.1. Appearance of two individual lines of callus tissue established from leaf tissue explants of P. rigida. Callus tissue was derived from wounded leaf tissue explanted to a modified M-S media as described in the text. Callus was maintained in the dark at room temperature and transferred aseptically at 14 day intervals.

a. Appearance of a white line of callus tissue from leaf explants of P. rigida.

b. Appearance of a red line of callus from leaf explants of P. rigida.

or used fresh. Callus was ground in a mortar to a slurry and then blended in a Waring blender for 15 s. The resulting slurry was then subjected to breakage in a French pressure cell at 500-750 psi and the cell debris removed by centrifugation at 15,000 rpm in an SS-34 rotor for 30 min. The supernatant fraction was filtered through Whatman (No. 3 MM) filter paper and applied to a CM-52 column (15 x 2.7 cm) equilibrated in 5 mM sodium phosphate buffer, pH 6.7 at room temperature. The column was then eluted at room temperature with a 450 mL linear gradient from 0 to 0.3 M NaCl in 5 mM sodium phosphate buffer. After elution, the individual fractions were assayed for absorbance at 280 and 310 nm and reactivity to antibody by ELISA. Protein containing fractions which were established to be reactive with either anti-PAP-I or anti- PAP-II were further examined by SDS-PAGE and EITB analysis.

Fractions identified by ELISA, SDS-PAGE and EITB analysis to contain either anti -PAP-I or anti-PAP-II reactive material were pooled. This resulted in two pools from the CM-52 chromatography, designated Pool 1 (PAP-I like or anti-PAP-I crm) (0.1 to 0.15 M NaCl) and Pool 2 (PAP-II like or anti-PAP-II crm) (0.15 to 0.18 M NaCl). Each pool was individually concentrated at room temperature to 7.5 mL in a 50 mL Amicon stirred flow cell on a YM-10 membrane at 25 psi. The retentate from this concentration step was then applied to an SG-75 column which had been previously equilibrated in 0.1 M ammonium bicarbonate buffer (pH 8.9). The column was 140x 2.5 cm and was eluted at a flow rate of 0.6 mL/min. Fractions obtained from this chromatographic step were assayed as previously described for absorbance and reactivity to antibody by ELISA, SDS-PAGE and EITB.

Immunocytochemical studies with callus tissue

Callus tissue of P. rigida, grown as described was fixed in 3% glutaraldehyde, rinsed and dehydrated to 70% EtOH in ddw. This fixed tissue was embedded in LRW plastic, and the plastic hardened at 60°C for 24 h. Thin sections (100 A) were obtained and placed onto nickel grids coated with Formvar film. The grids with sections were placed on a solution of sodium phosphate buffer (pH 7.2) containing 0.1 % Tween-20 and 1% ovalbumin (PB:Tw:OA) for 15 min. The grids were then floated on a solution containing anti-PAP-I (1:5000) or anti-PAP-II (1:5000) for 45 min, and then rinsed by floating on PB:Tw:OA 3x15 min each. The grids were then floated on a solution containing rabbit anti-goat IgG for 45 min and then washed as above. The grids were then floated on a solution of colloidal gold coupled to Protein-A for 45 min, washed as described, and then blotted dry. The sections were visualized (without post-staining) for the distribution of gold label in an HU-11 E transmission electron microscope.

Results

Fractionation of PAP forms from extracts of callus cultures

Figure 3.2 shows the typical elution profile for callus culture extract from CM-52 cation exchange resin. The monospecific and cross-reactive antibody preparations allowed me to screen each fraction for material reactive with antibodies directed against PAP-I, PAP-II or PAP-S. Anti-PAP-S did not detect any antigens from callus extracts. The reactivity (as measured by ELISA) with anti-PAP-I (a polyclonal antibody which showed cross reactivity with PAP-I, II and S) or monospecific anti-PAP-II IgG of individual fractions from the CM-52 elution is also demonstrated in Figure 3.2. The cross-reactive anti-PAP-I provided a convenient probe for detecting

Figure 3.2. CM-52 elution profile of Phytolacca callus cell extract. The column (15 x 2.7 cm) was developed with a 500 mL linear gradient to 0.3 M NaCl in 5.0 mM phosphate (pH 6.1) buffer at a flow rate of 1.0 mL/min. 6.1 mL fractions were collected and assayed for A_{280} (protein) and A_{410} (ELISA) as described in the text. —O—O—O— (protein, A_{280}), —□—□—□— (anti- PAP-I ELISA reactivity), —△—△—△— (anti-PAP-II ELISA reactivity).

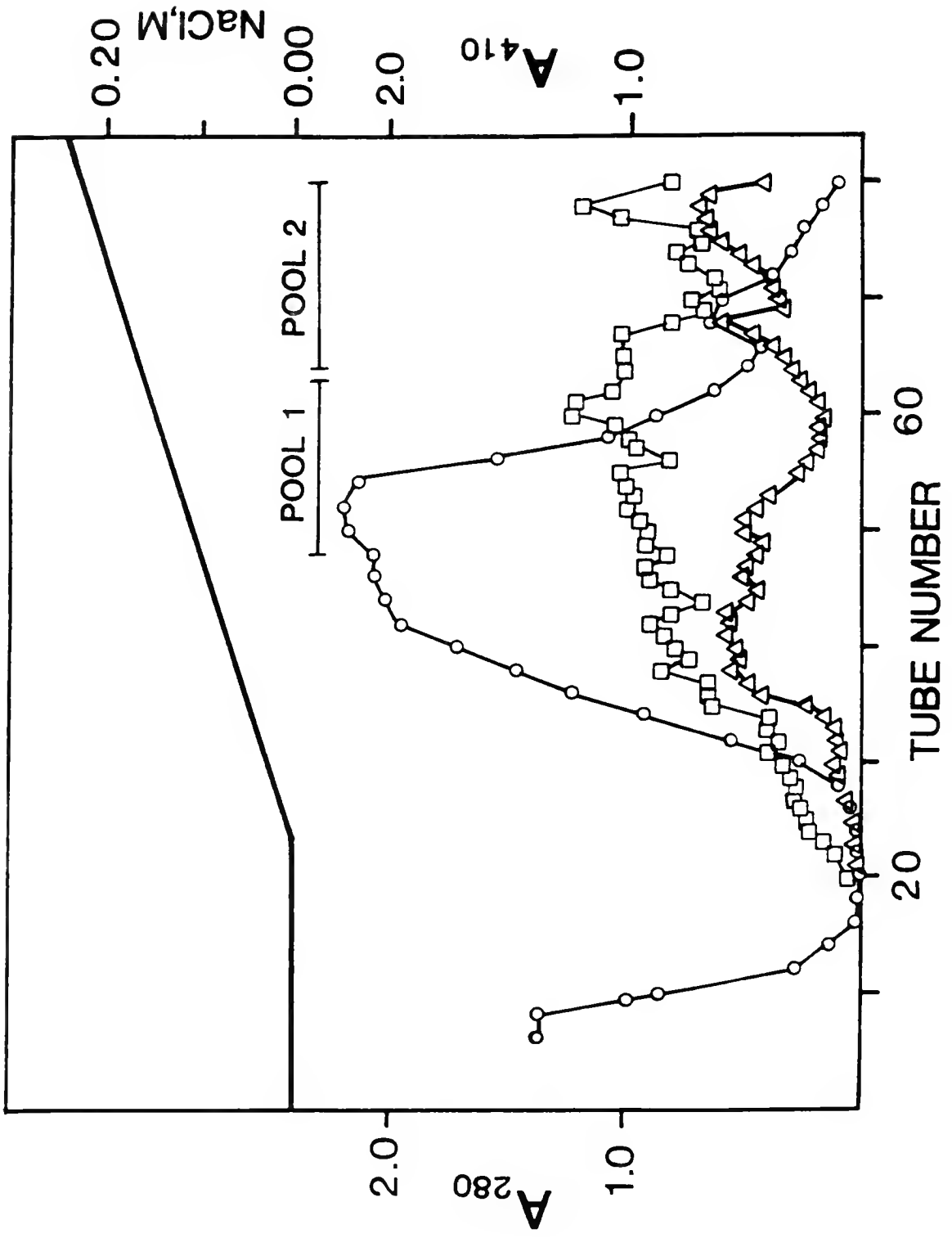
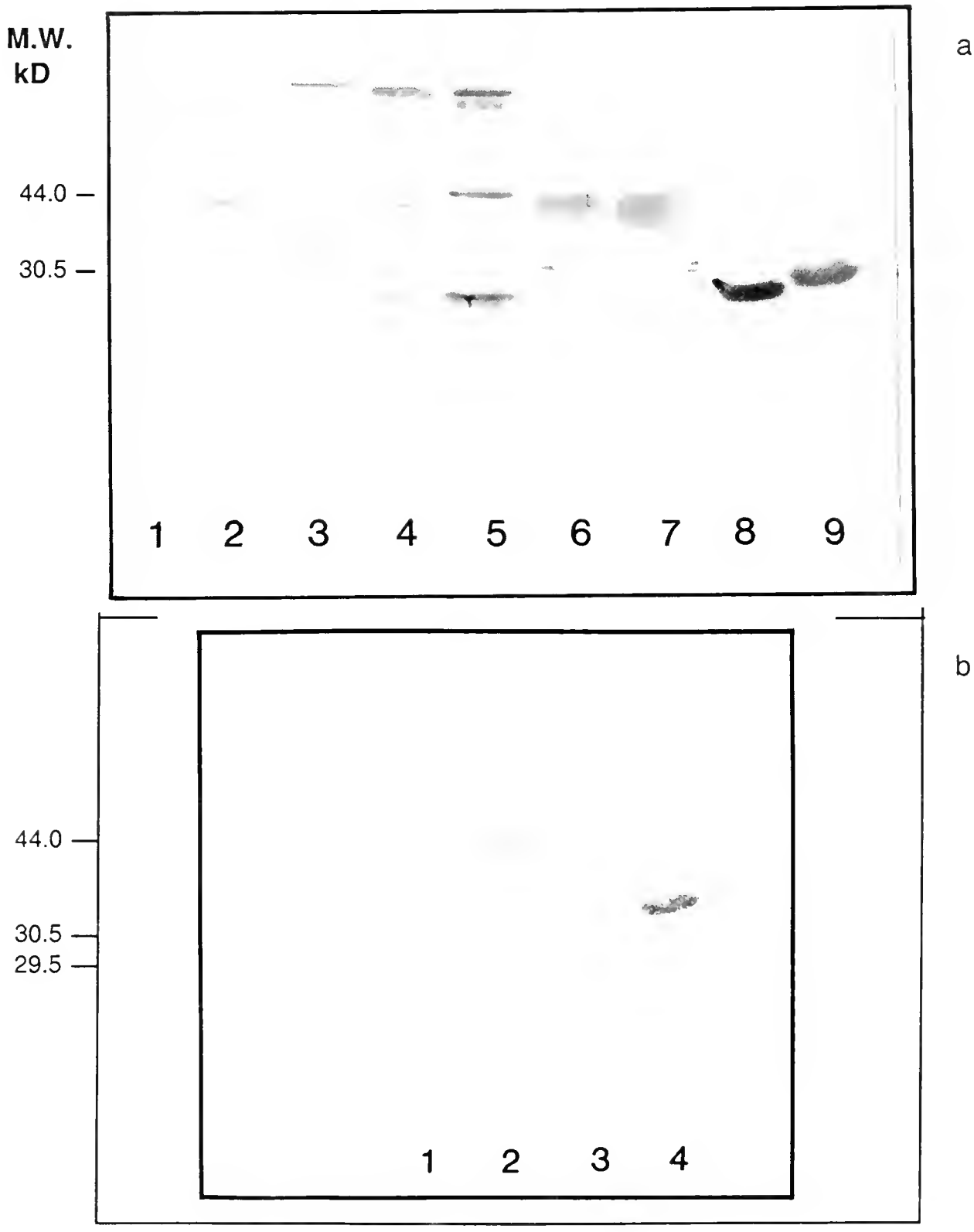

0.20
NaCl,M
0.00
2.0
A410
1.0
POOL 1
POOL 2
2.0
A280
1.0
20
60
TUBE NUMBER

Figure 3.3. EITB analysis of fractions from CM-52 chromatography of pokeweed callus extract. Individual tubes from the CM-52 fractionation of callus extract were selected from peak fractions (see Figure 3.2). 200 uL of each sample were boiled for three minutes in sample buffer and applied to a 12% polyacrylamide gel. Western blots were prepared as described in the text.

a. Lane 1: Crude extract of callus tissue after homogenization, breakage and centrifugation, 25 uL; Lanes 2-5: selected fractions (tube numbers 45, 50, 55 and 60) from Pool 1; Lanes 6–7: selected fractions (tube numbers 64 and 66) from Pool 2; Lane 8: PAP-I standard, 4 ug; Lane 9: PAP-II standard, 4 ug . The western blot was probed with anti-PAP-I antibody at a dilution of 1:1000.

b. same as for 2 a except the antibody used in the western blot was anti-PAP-II at a dilution of 1:1500. Lanes 1-4 of panel b correspond to Lanes 6-9 of panel a.


M.W.
kD
44.0
30.5
1 2 3 4 5 6 7 8 9
a
b
44.0
30.5
29.5
1 2 3 4

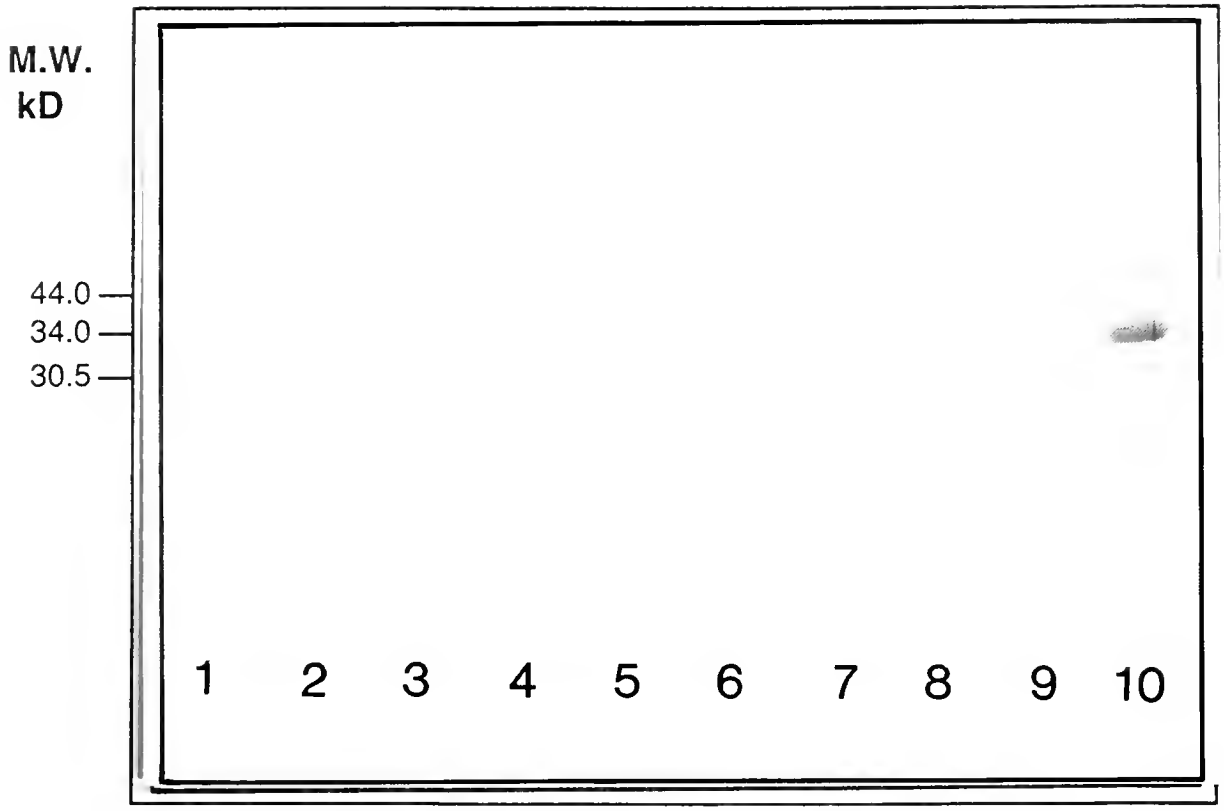


Figure 3.4. Western blot analysis of fractions from CM-52 chromatography of tissue culture extract demonstrating the reactivity of two protein bands with monospecific anti -PAP-II.

The western blot was prepared from 12% polyacrylamide gels as described. The separation performed here was identical in all conditions to that described previously in Figure 3.3. Fractions for SDS-PAGE were chosen from Pool 1 (containing anti-PAP-I crm) and from Pool 2 (containing anti-PAP-II crm). 200 uL of sample was electrophoresed unless otherwise indicated. Monospecific antibody to PAP-II was utilized at a dilution of 1:1500.

Lane 1: crude extract of callus tissue after homogenization, breakage and centrifugation, 25 uL; Lanes 2-5: selected fractions from Pool 1 (see chromatogram of Figure 3.2); Lanes 6-9: selected fractions from Pool 2 (see chromatogram of Figure 3.2); Lane 10: PAP-I and PAP-II standards, 4 ug each.

any PAP-related proteins present in the tissue culture extract. The presence or absence of protein species bearing PAP-II antigens or PAP-S antigens was then further examined by utilizing monospecific anti-PAP-II or monospecific anti-PAP-S. Anti-PAP-S detected no antigens in these analyses. Anti-PAP-I detected a heterogeneous peak of cross- reactive material eluting with 0.1 M NaCl, and two later peaks at 0.12 to 0.18 M NaCl. Analysis of individual fractions from the CM-52 chromatography by ELISA with anti-PAP-II specific antibody revealed that this antibody reacted with an early eluting peak at 0.12 to 0.15 M NaCl, corresponding to a similar pattern of reactivity seen with the anti-PAP-I antibody. In contrast to the peak detected by the anti-PAP-I antibody at tube number 60, anti-PAP-II showed a diminshed reactivity in this region of the profile. The anti-PAP-II antibody reacted with two later eluting components corresponding to a salt concentration of 0.18 M to 0.20 M (tube numbers 68-80).

Figure 3.3 demonstrates the reactivity of specific fractions from the CM-52 chromatography when analyzed by EITB. Figure 3.3 a shows the material reactive with cross-reactive anti-PAP-I antibody and Figure 3.2 b shows the material reactive with monospecific anti-PAP-II antibody. The specificity of this antibody for the PAP-II protein is evident from this blot. Lane 3, panel b, shows no reactivity of this antibody with PAP-I compared to the analogous lane 8 in panel a.

This analysis indicates that the peak eluting with 0.1 M NaCl, reactive with anti-PAP-I but not anti-PAP-II, (lanes 2-4, Fig.3.2 a) is composed of several discrete components of high molecular weight. A component with a molecular mass of 29.5 kD (lane 4-5, Fig. 3.3 a) eluted with 0.14 M NaCl and was nearly identical in size to native PAP-I isolated from spring leaf tissue. The material eluting later in the gradient at 0.18 M NaCl was comprised of a

single band, evident in this analysis which migrated with an apparent mass of 44 kD. Subsequent analyses demonstrated that two proteins were contained in Pool 2, one with an apparent mass of 44 kD and the other with a mass of 34 kD. This is demonstrated in the Western blot depicted in Figure 3.4. Figure 3.4 is analogous to Figure 3.3 in that it depicts discrete fractions from a CM-52 fractionation of tissue culture extract subjected to SDS-PAGE and western blotting. The appearance of two diffuse protein species present in Pool 2 from CM-52 chromatography when detected by monospecific anti-PAP-II antibody is shown in Figure 3.4. These two bands were uniquely detected by monospecific anti-PAP-II (Fig. 3. 3b, lanes 1,2; Figure 3.4, lanes 8-9) while no band was detected at the position expected for native PAP-II (lane 4, Figure 3.3 b) isolated from summer leaf tissue. Note that the diffuse nature of this material after SDS-PAGE and Western blot analysis makes it difficult to assign a discrete molecular weight. This higher apparent molecular weight for the anti-PAP-II crms and their diffuse appearance on SDS gels when compared to PAP-II from summer leaf tissue suggested that they might represent a form of PAP-II modified by glycosylation and/or possessing additional peptide sequence. Carbohydrate was present in the fractions comprising this peak (see below).

The fractions from the CM-52 chromatography containing anti-PAP-I reactive material were pooled and designated, Pool 1. Those fractions reactive with anti-PAP-II were pooled and designated, Pool 2. These pools were independently concentrated to 7.5 mL and applied to an SG-75 column and eluted with 0.1 M NH_4CO_3. The results from the gel filtration of Pool 1 and Pool 2 are given in Figure 3.5 a and Figure 3.5 b, respectively. As is evident from Figure 3.5 a, gel chromatography on SG-75 resolved Pool 1 into 5 peaks identified by A_{280}.

Figure 3.5. SG-75 gel sieving chromatography elution profiles for Pool 1 (containing anti-PAP-I cross-reactive proteins) and Pool 2 (containing anti-PAP-II cross-reactive proteins) obtained by CM-52 chromatography of callus tissue extract as described. The column (140 x 2.5 cm) was developed with 0.1 M NH_4CO_3 (pH 8.6) at a flow rate of 0.6 mL/min. 6.0 mL fractions were collected and assayed for A_{280} (protein) and A_{405} (ELISA) as described in the text.

—○—○—○— (Protein, A_{280})
—□—□—□— (ELISA reactivity (A_{405}); anti-PAP-I, 1:1000)
—△—△—△— (ELISA reactivity (A_{405}) ; anti-PAP-II, 1:1500)

a. SG-75 gel sieving chromatography elution profile of Pool 2

b. SG-75 gel sieving chromatography elution profile of Pool 1

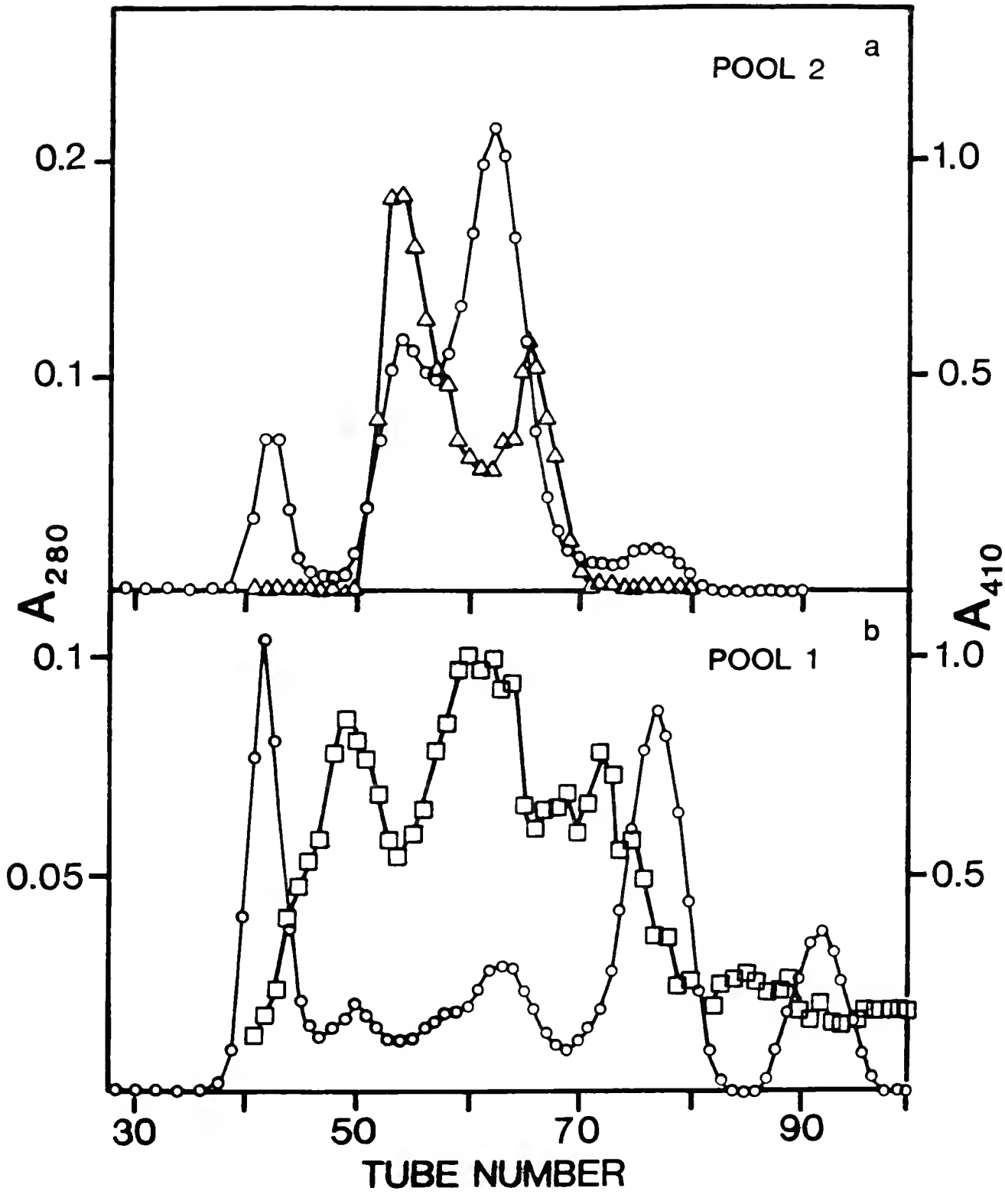
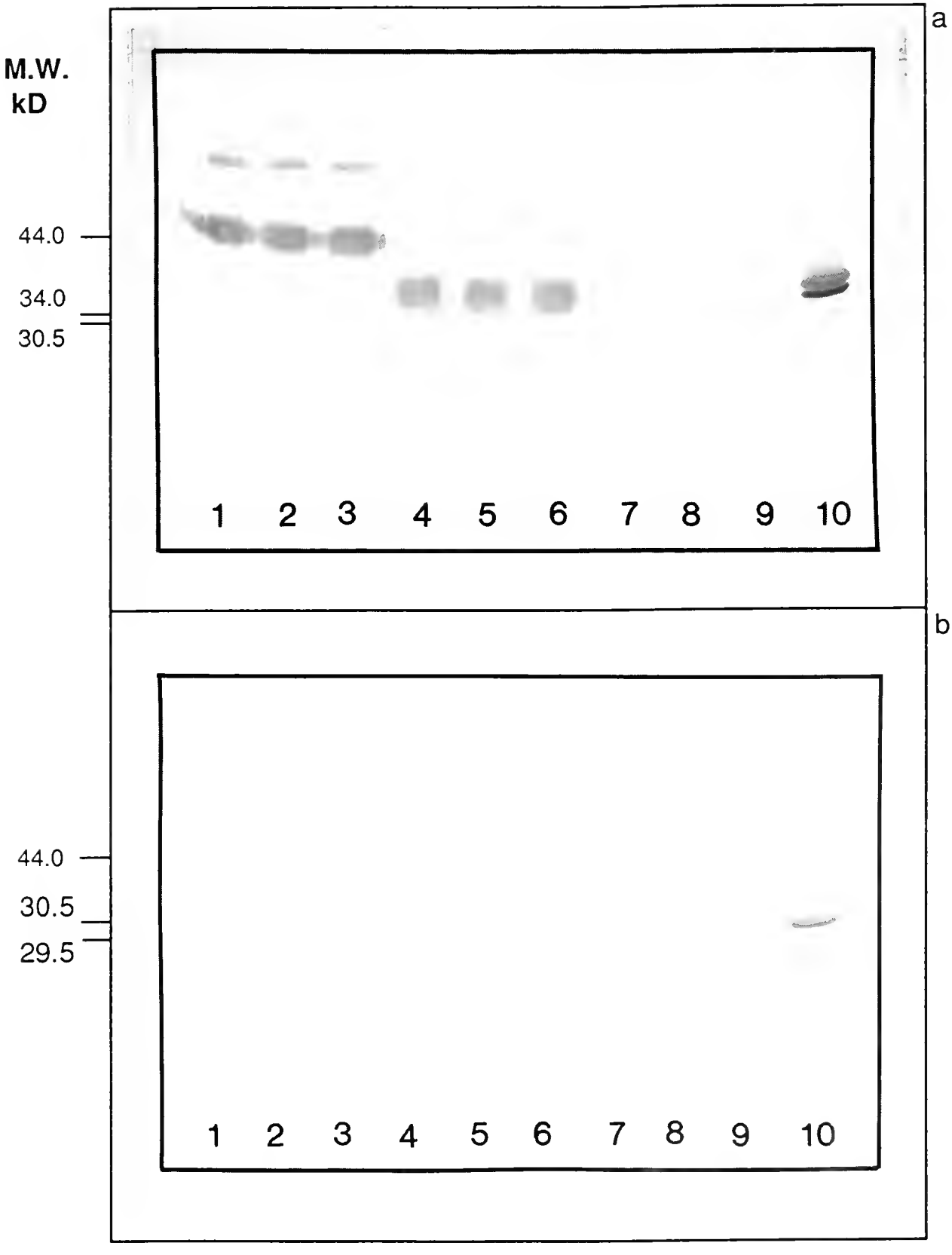

a
POOL 2
0.2
0.1
1.0
0.5
A_{280}
A_{410}
b
POOL 1
0.1
0.05
1.0
0.5
30
50
70
90
TUBE NUMBER

Figure 3.6. EITB analysis of fractions from SG-75 chromatography of Pool 1 and Pool 2 from CM-52 chromatography of callus extract.

a. Lanes 1-3: tubes 51-53 (peak 1) from SG-75 gel chromatography of Pool 2 (anti-PAP-II crm, see Figure 3.2); Lanes 4-6: tubes 62-64 (peak 2) from SG-75 gel chromatography of Pool 2; Lanes 7-9: tubes 76-78 (peak 4, anti-PAP-I crm) from SG-75 gel chromatography of Pool 1; Lane 10: PAP-I and PAP-II standards of 4 ug each. The blots were prepared as described. Anti-PAP-I antibody cross-reactive with PAP-II and PAP-S was utilized at a dilution of 1:1000 to screen extracts.

b. Same as for the above except that the blot was developed with anti-PAP-II antibody (monospecific) at a dilution of 1:1000. Blots were prepared as described previously.


a
M.W.
kD
44.0
34.0
30.5
1 2 3 4 5 6 7 8 9 10
b
44.0
30.5
29.5
1 2 3 4 5 6 7 8 9 10

Peak 4 corresponds to PAP-I with an apparent molecular weight of 29.5 kD (based on elution position from SG-75) while peaks 1-3 are higher molecular weight species which are reactive with this anti-PAP-I antibody. Pool 2 (Figure 3.5 b) containing anti-PAP-II reactive material, was resolved into three distinct peaks on SG-75, two of which were reactive with anti-PAP-II antibody.

Figure 3.6 shows the reactivity of specific fractions from SG-75 of Pool 1 and Pool 2 when analyzed by EITB. Figure 3.6a demonstrates the reactivity to the anti-PAP-I antibody and Figure 3.6b shows the reactivity to the anti-PAP-II antibody.

Chemical properties

Both peak fractions 1 and 2 from Pool 2 and 1-4 from Pool 1 were assayed for total carbohydrate. Table 3.1 shows the relative amounts of protein and carbohydrate for the various fractions. The carbohydrate associated with the two anti-PAP-II cross reactive proteins was further characterized by paper chromatography. These two compounds were individually hydrolysed in mineral acid for one h, and the hydrolysate was chromatographed as described. The mobilities of the released monosaccharides were compared to the Rfs of pure standards of glucose and mannose, which indicated the presence of glucose and mannose as the only saccharides (data not shown). On the basis of relative spot intensity glucose accounted for greater than 90% of the total carbohydrate. The glucose was further quantified by an enzyme coupled analysis as described. By this analysis, glucose accounted for only 15-25% of the total CH_2O detected by the phenol-sulfuric acid assay which raised the question of the accuracy of the colorimetric assay when applied to these glycoproteins.

To identify specific carbohydrate containing proteins, the reactivity of these fractions to the lectin ConA was examined on Western blots. Individual fractions from both CM-52 and SG-75 chromatography were shown to be reactive with ConA, following EITB. Figure 3.7 shows the results of one such analysis. Ovalbumin and RTA (lanes 1 and 2) are strongly reactive while PAP-I and PAP-II (lane 3) are not reactive, indicating that the procedure does correctly detect glycoproteins after electrophoretic transfer. Lanes 4-6 represent fractions from CM-52 ion exchange chromatography which correspond to fractions reactive with anti-PAP-I (see Figure 3.3 a). By this analysis one may infer the presence of glycosylated, ConA reactive species of relatively high molecular weight (greater than 30 kD with a prominent band at 50 kD), bearing anti-PAP-I epitopes. Lanes 7-10 correspond to CM-52 fractions reactive with anti-PAP-II, while lane 11 is a concentrated pool of anti-PAP-II crm with a mobility corresponding to a polypeptide with a molecular weight of 34 kD. The anti-PAP-II crm of 34 kD (lanes 7, 8) is clearly reactive with ConA, while the material with a greater molecular weight of 44 kD (lanes 9,10) is detected as a blanched (negative staining) component apparently giving an anomalous reaction with ConA and/or anti-ConA-alkaline phosphatase. Such a negative staining around a reactive species by a lectin blot analysis has been observed by Faye and Chrispeels (1985) and is ascribed to an overabundance of the transferred protein. Lane 12 is a concentrated Pool I (containing anti-PAP-I crm from CM-52 fractionation) prior to SG-75 gel sieving chromatography while lanes 13 and 15 are purified anti-PAP-I crm of molecular weight 29.5 kD after SG-75 gel sieving. These lanes correspond to peak 4 , Figure 3.4 a, which is strongly reactive with anti-PAP-I. This PAP-I like material is clearly non-reactive with ConA except for high molecular weight components which may

Table 3.1. Carbohydrate and protein composition of PAP proteins from callus tissue.

Starting Material	Protein (mg)	Glucose equivalents (mg)	CH_2O/Protein (gm/gm)
Pool 1 CM-52	3.38	3.04	0.99
Fraction1 SG-75	0.39	0.69	1.7
Fraction2 SG-75	0.39	0.42	1.1
Fraction3 SG-75	0.51	0.45	0.88
Fraction4 SG-75	1.62	0.15	0.09
Pool 2 CM-52	8.00	11.34	1.4
Fraction1 SG-75	2.07	3.09	1.49
Fraction2 SG-75	2.25	0.81	0.36

Figure 3.7. EITB analysis of ConA binding to pokeweed callus tissue proteins.

Gel electrophoresis and western blotting of samples were performed on 12% polyacrylamide gels as described in the text. A 100 uL aliquot of the appropriate sample was applied per well. Following transfer the blots were treated with ConA at 0.1 ug/mL of buffer, followed by antibody at the appropriate dilution as described in the text.

Lane 1: 5ug ovalbumin; Lane 2: ricin A chain (RTA), 5 ug; Lane 3: 5 ug of PAP-I and 5 ug of PAP-II from green leaf tissue; Lanes 4-8: selected fractions from CM-52 chromatography of callus tissue extract; Lane 9: Pool 2 (anti-PAP-II crm) from CM-52 chromatography; Lane 10: Pool2-Peak 1 (44kD anti-PAP-II crm) from SG-75 chromatography of CM-52 fractionated callus extract; Lane 11: Pool 2-Peak 2 (34 kD anti-PAP-II crm) from SG-75 chromatography of CM-52 fractionated callus extract; Lane 12: Pool 2 (anti-PAP-II crm) from CM-52 prior to chromatography on SG-75; Lane 13: Pool 1, peak 4 (anti-PAP-I crm with an M_r of 29,000) after SG-75 gel sieving chromatography; Lane 14: Pool 1 (anti-PAP-I crm) from CM-52 prior to chromatography on SG-75; Lane 15: as for lane 13.

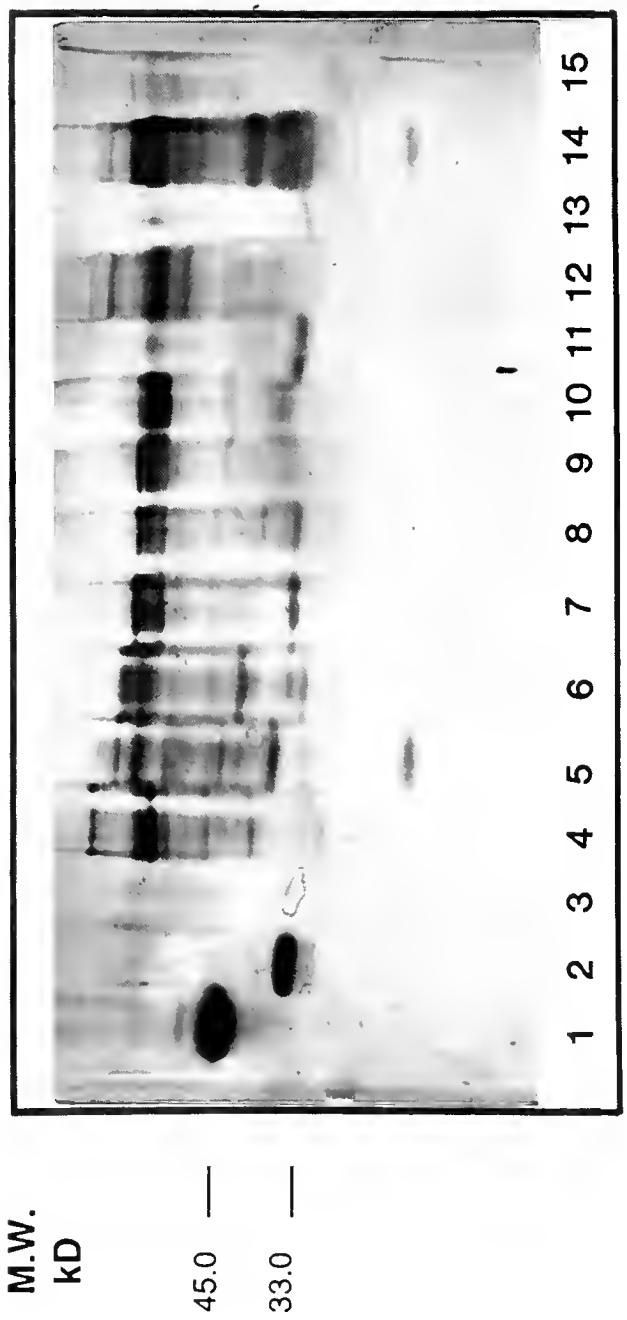

M.W.
kD
45.0
33.0
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15

Table 3.2. Summary of reactivities of tissue culture derived proteins with anti-PAP-I, anti-PAP-II and ConA with specific reference to Figure 3.6.

Protein Fraction	Lane # (Figure 3.6)	Mol. Wt (kD)	Reactivity		
			ConA	anti-PAP-I	anti-PAP-II
Ovalbumin	1	45	+	-	-
Ricin A Chain	2	33	+	-/+	-
PAP-I	3	29.5	-	+	-
PAP-II	3	31.0	-	-	+
Tissue Culture					
Pool 1 Fractions (anti-PAP-I crm)	4-6	>30.0	+	-	+
Pool 1 (concentrate)	12	>30.0	+	-	+
Purified Peak 4 (anti-PAP-I crm)	13,15	29.5	-	+	-
Pool 2 Fractions (anti-PAP-II crm)	7,8	34-44	+	-	+
Pool 2 (concentrate)	14	34-44	+	-	+
Peak 1, Pool 2	9,10	44	+	-	+
Peak 2, Pool 2	11	34	+	-	+

represent glycosylated precursors (see above). Lane 14 is a concentrated pool of anti-PAP-II crm from CM-52 chromatography prior to gel filtration and demonstrates the reactivity of this pool with ConA. Of interest is the presence in all of these fractions of a large molecular weight component of about 55 kD which is intensely reactive with the ConA probe and which is detected by anti-PAP-I antibody (see Figure 3.5 a, Lanes 1-3).

It is important to emphasize that in control experiments this reactivity was blocked by the presence of 0.3 M alpha-D methylmannopyranoside (a specific competitor for ConA binding) during the reaction with the ConA (data not shown). The data discussed above relating to Figure 3.6 is summarized in Table 3.2. This Table relates the reactivities of the individual fractions discussed from Figure 3.6 to anti-PAP-I, anti-PAP-II and Con A.

Amino terminal sequence data

Amino terminal sequence data for the two forms of the anti-PAP-II cross-reactive proteins from callus tissue have been obtained. Both Peak 1 (44 kD) and peak 2 (34 kD) share the following (concensus) amino terminal sequence (first ten): ser-his-his-his-arg-?-his-his-his-arg-. This sequence is different from the known sequence for PAP-II and it suggests that these proteins may carry a peptide sequence which is involved in the targetting or in the processing of these proteins in vivo (see Chapter 4).

Translation inhibitory activities

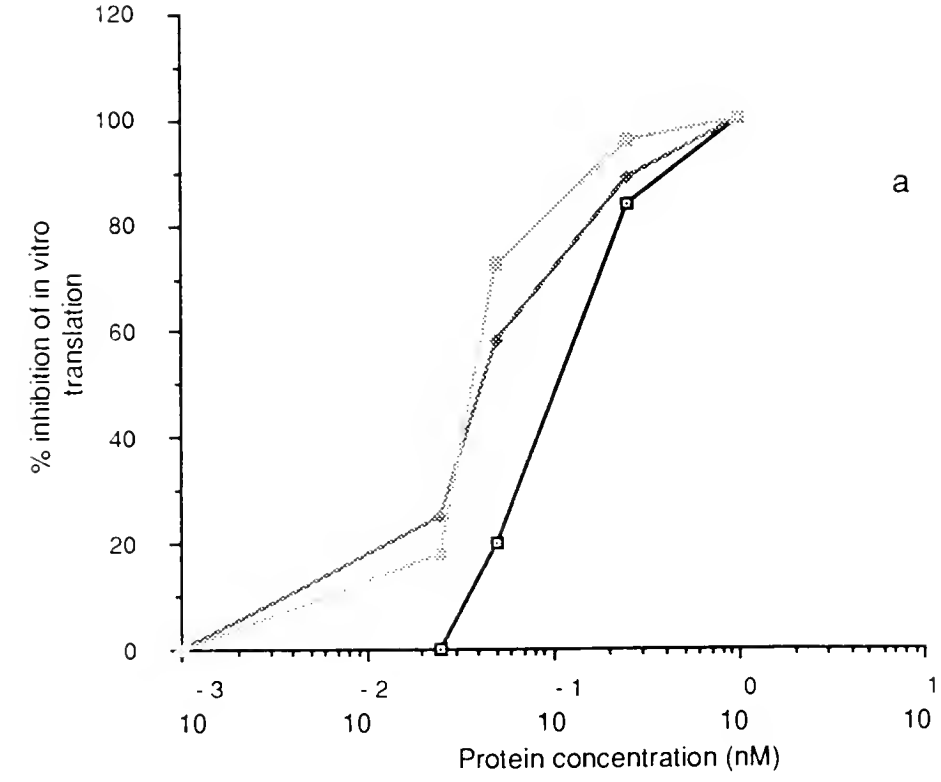
These proteins from callus tissue, the PAP-II like material (Peaks 1 and 2, Pool 2, Figure 3.4 b) and the PAP-I like material (peak 4, Pool 1, Fig. 3.4 a) were assayed for their inhibitory effect towards in vitro translation (in a wheat germ based system) and were compared to the activity of standard PAP proteins isolated from the spring and summer leaf tissue and seed. The results of this assay are presented in Figure 3.8 and are summarized in

Figure 3.8. Demonstration of the ability of the PAP proteins from leaf, seed and callus tissues of P. rigida to inhibit an in vitro translation assay utilizing wheat germ extract.

PAP proteins, PAP-I, PAP-II and PAP-S and the tissue culture proteins were assayed for their ability to inhibit the incorporation of ^{3}H-leucine into an acid precipitable fraction. Assays were performed by pre-incubating 10 uL of the inhibitor protein with 10 uL of wheat germ as described in the text. After addition of mRNA, translation was allowed to proceed for 40 min, and the reaction stopped. An acid insoluble fraction was collected from the reaction mix and collected on a glass fiber filter. Following drying, the samples were measured for the incorporation of radioactive label by liquid scintillation counting as described.

a. Effects of increasing amounts of PAP-I (—⊡—), PAP -II (—◆—) and PAP-S(—□—) on in vitro translation activity, measured as a percent of inhibition.

b. Effect of increasing amounts of anti-PAP-I crm (29.5 kD —⊡—), and the anti-PAP-II crms, Peak 1 (44kD , —◆—) and Peak 2 (34 kD, —□—) on in vitro translation activity, measured as a percent of inhibition.


120
100
80
60
40
20
0
% inhibition of in vitro translation
a
- 3
10
- 2
10
- 1
10
0
10
1
10
Protein concentration (nM)

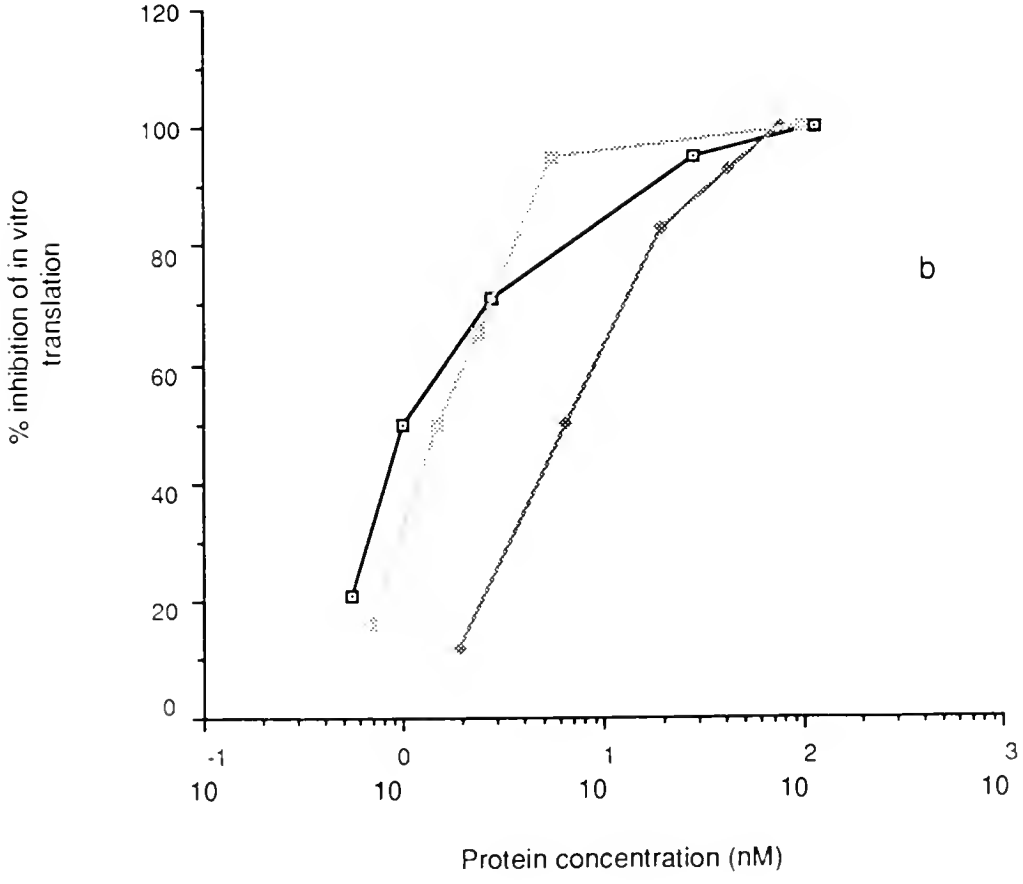

120
100
80
60
40
20
0
% inhibition of in vitro translation
b
-1
10
0
10
1
10
2
10
3
10
Protein concentration (nM)

Table 3.3 In vitro translation inhibitory activities for PAP proteins from leaf, seed and callus tissue.

Protein Sample	IC-50[a] nM	Inhibitory activity relative to PAP-I
Leaf		
PAP-I	0.085	1.0
PAP-II	0.045	0.53
Seed		
PAP-S	0.040	0.47
Callus		
Pool1 Peak4 (29.5 kD anti-PAP-I crm)	1.0	11.7
Pool2 Peak1 (44 kD anti-PAP-II crm)	6.4	75.3
Pool2 Peak2 (34 kD anti-PAP-II crm)	1.5	17.6

a. IC-50 values were determined from the data presented in Figure 3.7

Table 3.3. The IC_{50} values of the mature PAP proteins are similar to each other, as observed in previous studies (Preston and Ervin, 1987). The PAP-II like proteins are 142-fold (44 kD fraction) and 33-fold (34 kD fraction) less inhibitory than the mature PAP-II isolated from leaf tissue. The PAP-I like protein is approximately 20-fold less inhibitory than PAP-I isolated from leaves.

Immunocytochemical localization of anti-PAP-I crm and anti-PAP-II crm in callus tissue

The results of the immunogold labelling studies with the callus tissue are shown in Figure 3.9. The PAP-I like material immuno-reactive with anti-PAP-I is distributed to the cell wall matrix of the callus cells (panel b) while the PAP-II like material is distributed to the cytoplasmic space next to the cell wall. The label occurs in intracellular clusters and may be compartmentalized in vesicles. Some label is wall associated. The contrast of the cell wall material in these sections is low because the tissue was not poststained prior to visualization.

SDS-PAGE of CNBr fragmented PAP proteins

Based upon the number of methionines in pure PAP forms from P. americana one would expect 5, 9 and 7 polypeptides (n+1), for PAP-I, II (Irvin et al., 1980) and S (Barbieri et al., 1982) , respectively. Figure 3.10 demonstrates the pattern obtained from the CNBr mediated cleavage of PAP-I, PAP-II and PAP-S and the tissue culture proteins which are cross-reactive with anti-PAP-II when analysed by SDS-PAGE. PAP-S(Lane 2) shows 5 major fragments with the predominant fragments at 18 kD, 8.5 kD and 6.6 kD. PAP-I (Lane 3) was fragmented to yield 5 major fragments at 18 kD, 13.5 kD, 9 kD, 7.5 kD and 6.6 kD. The PAP-II fragments (Lane 8) are present as 5 major peptides with a triplet of bands

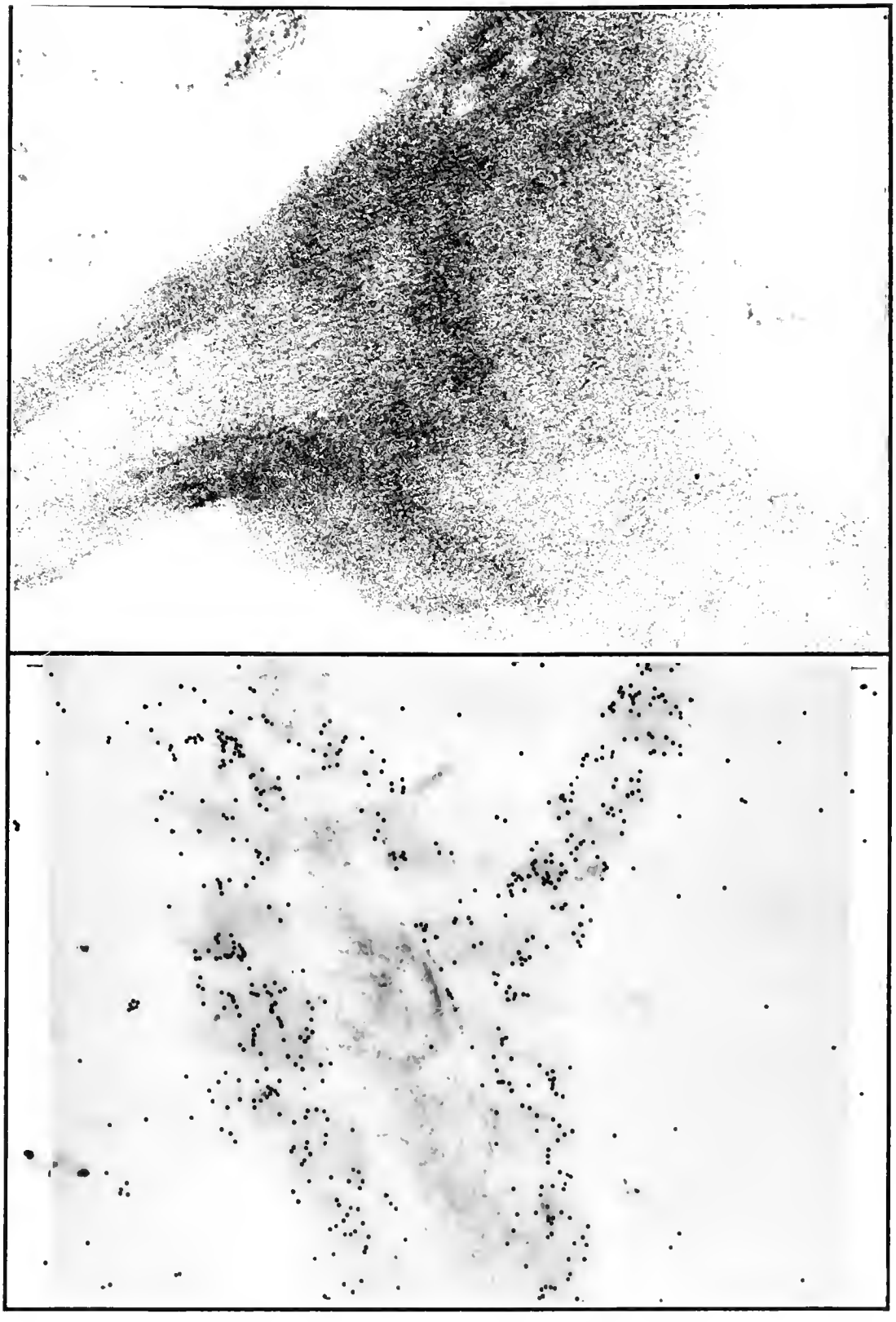
Figure 3.9. Immunocytochemical localization of cross-reactive proteins in thin sections of callus tissue from P. rigida utilizing anti-PAP-I and anti-PAP-II monospecific probes.

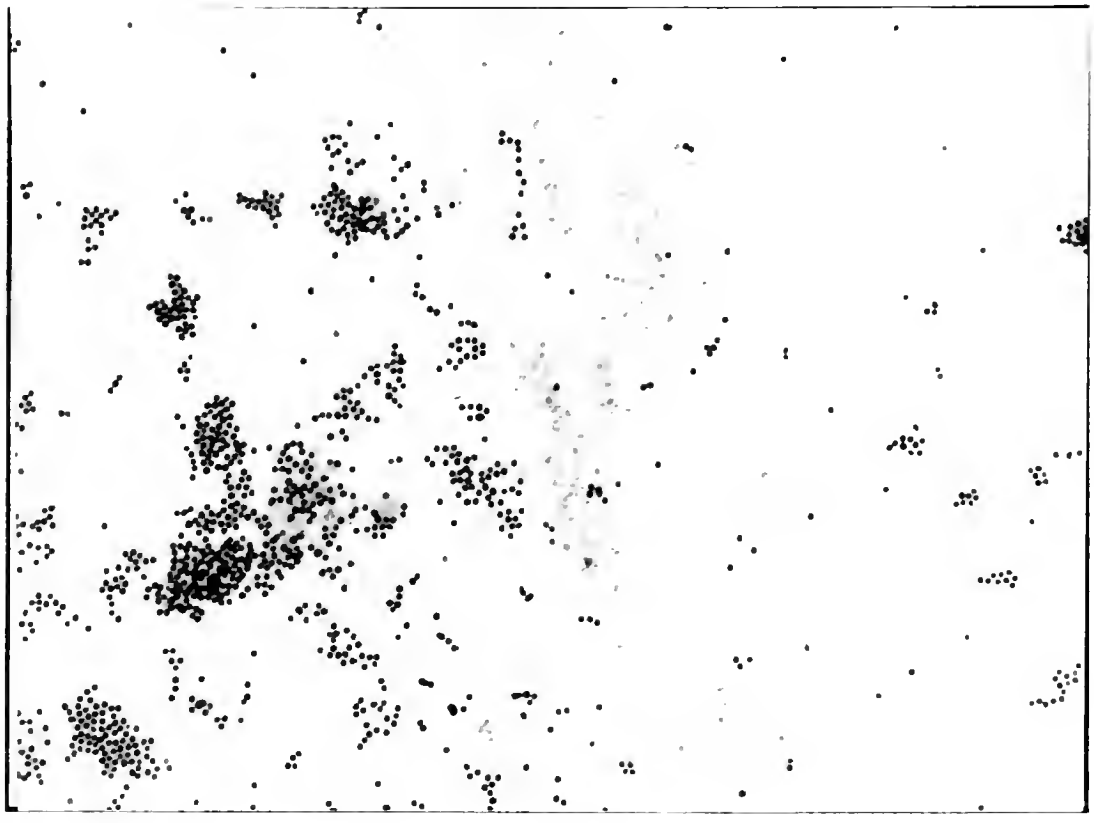
Thin sections of callus tissue were obtained from callus tissue which had been fixed, dehydrated and embedded in LRW resin. Sections of 100 A were obtained by cutting on an LKB microtome utilizing a diamond knife. The sections were placed onto Formvar coated nickel grids and stained with antibody as described in the text. Bound antibody was detected with an 18 nm colloidal gold probe coupled to protein A and visualized in an HU-II E transmission electron microscope.

a. Wall tissue of callus probed with a non-immune goat serum (1:5000 dilution).

b. Wall tissue of callus probed with monospecific anti-PAP-I (1:1000 dilution).

c. Wall tissue of callus probed with monospecific anti-PAP-II (1:1000 dilution).


a
b



c

Figure 3.9 (continued)

at 11.5 kD, 9.5 kD and at 8.5 kD. It is clear from this analysis that the different PAP proteins share few cleavage products in common. This same Figure (Lanes 5, 6, 7, and 9) demonstrates the polypeptide products obtained by CNBr hydrolysis of the proteins isolated from tissue culture extracts which are cross-reactive with antibody to PAP-II from leaf tissue. Lanes 5 and 6 demonstrate the fragmentation products obtained from the Peak 1 protein (44kD) in two separate treatments from two separate protein isolations and lanes 7 and 9 are the Peak 2 protein (35 kD) after fragmentation. When compared to the fragmentation pattern obtained after SDS-PAGE of PAP-II, there are obvious differences. In particular, with the tissue culture derived proteins, a large fragment with a molecular weight of 13.5 kD accumulates and is apparently resistant to further cleavage. If one examines carefully the bands at position 8.5 kD to 11.5 kD (Lanes 5-9) it appears that there are homologous bands at those positions. It is as though the CNBr mediated hydrolysis of the tissue culture derived proteins is inhibited. PAP-II, by contrast is fragmented to yield 5 major polypeptides on SDS-PAGE, all with a mobility less than this large fragment of 13,500. There is a faint band in Lane 8 at the 13.5 kD position corresponding to that observed with the tissue culture proteins. This suggests that PAP-II does yield this fragment after CNBr attack, but that the fragment is further cleaved. Formic acid is capable of cleaving peptide bonds at Asp-Pro bonds and may introduce anomalies when analyzing CNBr cleavage products. However, there is no apparent mobility shift of PAP proteins after incubation in 70% formic acid.

Western blot analysis of these proteins utilizing antibody directed against PAP-II, Peak 1 (44 kd polypeptide) from tisssue culture and peak 2 (35 kD

polypeptide) from tissue culture is presented in Figure 3.10. The reactivity of the peptides obtained by CNBr digest reveals that there are epitopes shared between PAP-II and the tissue culture derived proteins, but this reactivity is complex. In particular, anti-PAP-II reacts with PAP-II (Lane 2) and the CNBr treated protein (Lane 3). The reactivity with the CNBr fragments (Lane 3) is obscured by the appearance of this preparation as a 'streak' following electrophoresis, but major bands of reactivity occur at molecular weights of 7.5 kD, 9.8 kD, 12 kD and 15.5 kD. Lane 5 is intact 44 kD protein and cross-reacts with this antibody. Lane 6 is the fragmentation pattern after CNBr treatment of the 44kD protein and demonstrates that this antibody recognizes a major epitope associated with the fragment of 13.5kD and a fragment with a mobility very close to this. There is a fragment which shows weak reactivity at 9.8 kD. The Peak 2 protein (Lane 8) with a molecular weight of 34 kD and its fragmentation products (Lane 9) react similarly. The three major peptide fragments recognized by anti-PAP-II have molecular weights of13.5 kD (but does not react with the closely associated fragment at 15 kD, as with the Peak 1 material), 10.5 kD , and 9.8 kD.

The detection of peptides with antibody to the Peak 2 (34 kD) protein is shown in panel b. In contrast to the antibody described below (anti-Peak 1), this antibody does weakly detect PAP-II on a blot (Lane 2) and does detect fragments (Lane 3) from the CNBr digest of PAP-II. The peptides are not recognized as distinct bands, but rather as a blur with a lower limit at 19.0 kD, and then lower fragments at positions corresponding to 13 kD, 11 kD and 9.8 kD. This antibody reacts intensely with the Peak 1 protein (Lane 5) and detects major fragments from the CNBr digest of that protein at positions corresponding to 13.5 kD, 15 kD and 19 kD. Lane 8 shows the detection of native Peak 2 protein and Lane 9 shows the detection of CNBr fragment

Figure 3.10. SDS-PAGE analysis of polypeptides derived from the CNBr fragmentation of PAP proteins from leaf tissue and PAP-II like proteins from callus tissue of P. rigida.

Proteins to be fragmented were dialysed and lyophilized from deionized distilled water. Proteins (1.0 mg each) were treated with a 100-fold molar excess of CNBr to methionine as described. CNBr treated proteins were repeatedly lyophilized from ddw and resuspended in 1.0 mL of ddw. SDS-PAGE analysis was performed utilizing the low molecular weight SDS system of Giulian et al. (1985) as described in the text. Samples for electrophoresis were boiled for 90 sec in sample buffer (15 mM dithiothreitol, 1.5 M urea, 30% (w/v) sucrose, 1.0 % (w/v) SDS in 0.06 M tris-HCl, pH 6.8) and applied to the gel. 5- 10 uL of sample was applied per lane.

Lane 1. 44 kD anti-PAP-II crm (Peak 1) from callus tissue.
Lane 2. CNBr treated PAP-S.
Lane 3. CNBr treated PAP-I.
Lane 4. Blank.
Lane 5. CNBr treated 44 kD anti-PAP-II crm (Peak 1).
Lane 6. CNBr treated 44 kD anti-PAP-II crm (Peak 1) from a second isolation.
Lane 7. CNBr treated 34 kD anti-PAP-II crm (Peak 2).
Lane 8. CNBr treated PAP-II.
Lane 9. CNBr treated 34 kD anti-PAP-II crm (Peak 2) from a second isolation.
Lane 10. Molecular weight standards- Myoglobin CNBr digest

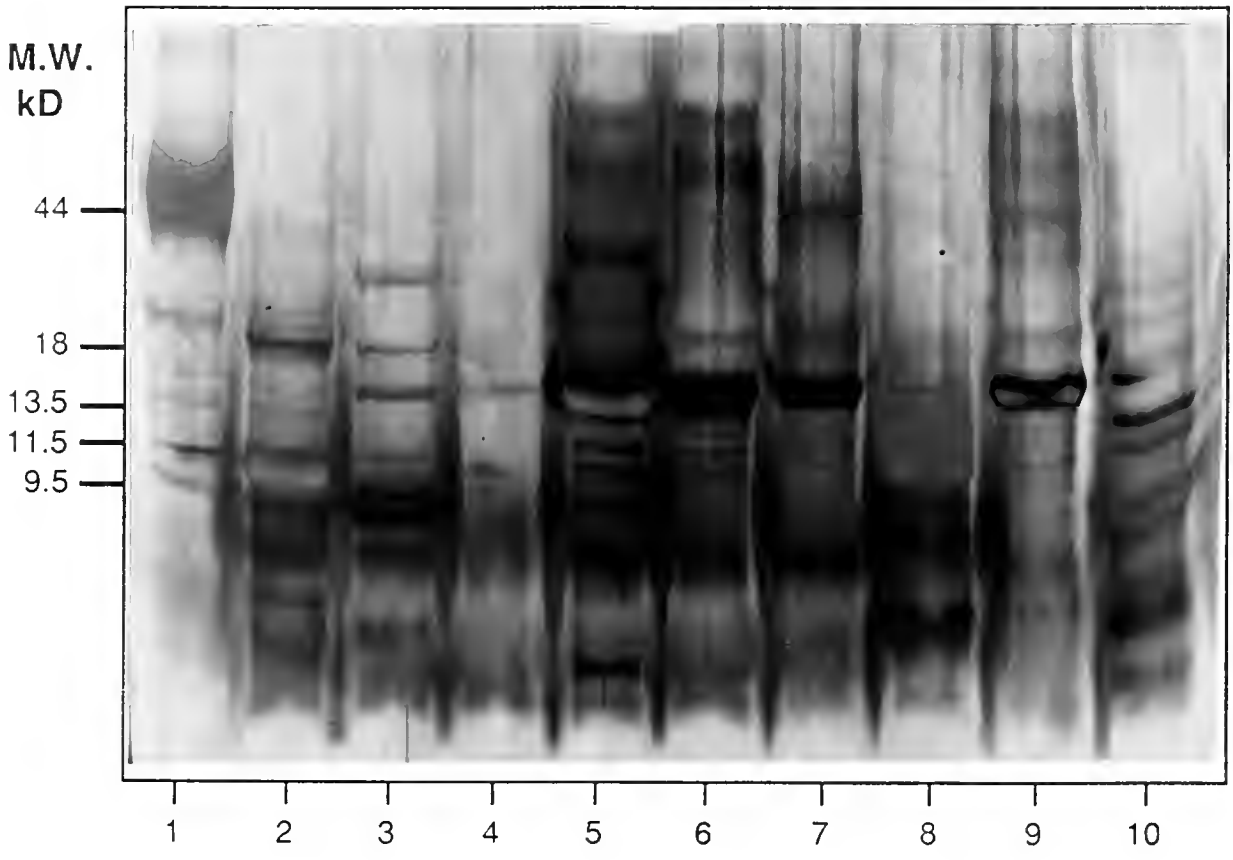

M.W.
kD
44
18
13.5
11.5
9.5
1
2
3
4
5
6
7
8
9
10

Figure 3.11. Western blot analysis of polypeptides obtained from the CNBr fragmentation of PAP-II from leaf tissue and anti-PAP-II crms (Peak 1 and Peak 2) from callus tissue of P. rigida.

CNBr fragmented proteins were obtained as described and electrophoresed in 12 % polyacrylamide gels following solubilization in sample buffer. 5-10 uL of protein were electrophoresed in each case. For the resolution and retention of small fragments in the gel system, 0.1 M sodium acetate was incorporated into the anode buffer of the electrophoretic system as recommended by Christy et al. (1989). Gels were transferred to nitrocellulose for one h at room temperature, and following transfer, placed into blocking buffer (PBS:Tw: 5% Carnation instant milk powder) for one h. Blots were then treated with antibody as described in the text and the color was developed from the oxidation by alkaline phosphatase of BCIP and the precipitation of NBT.

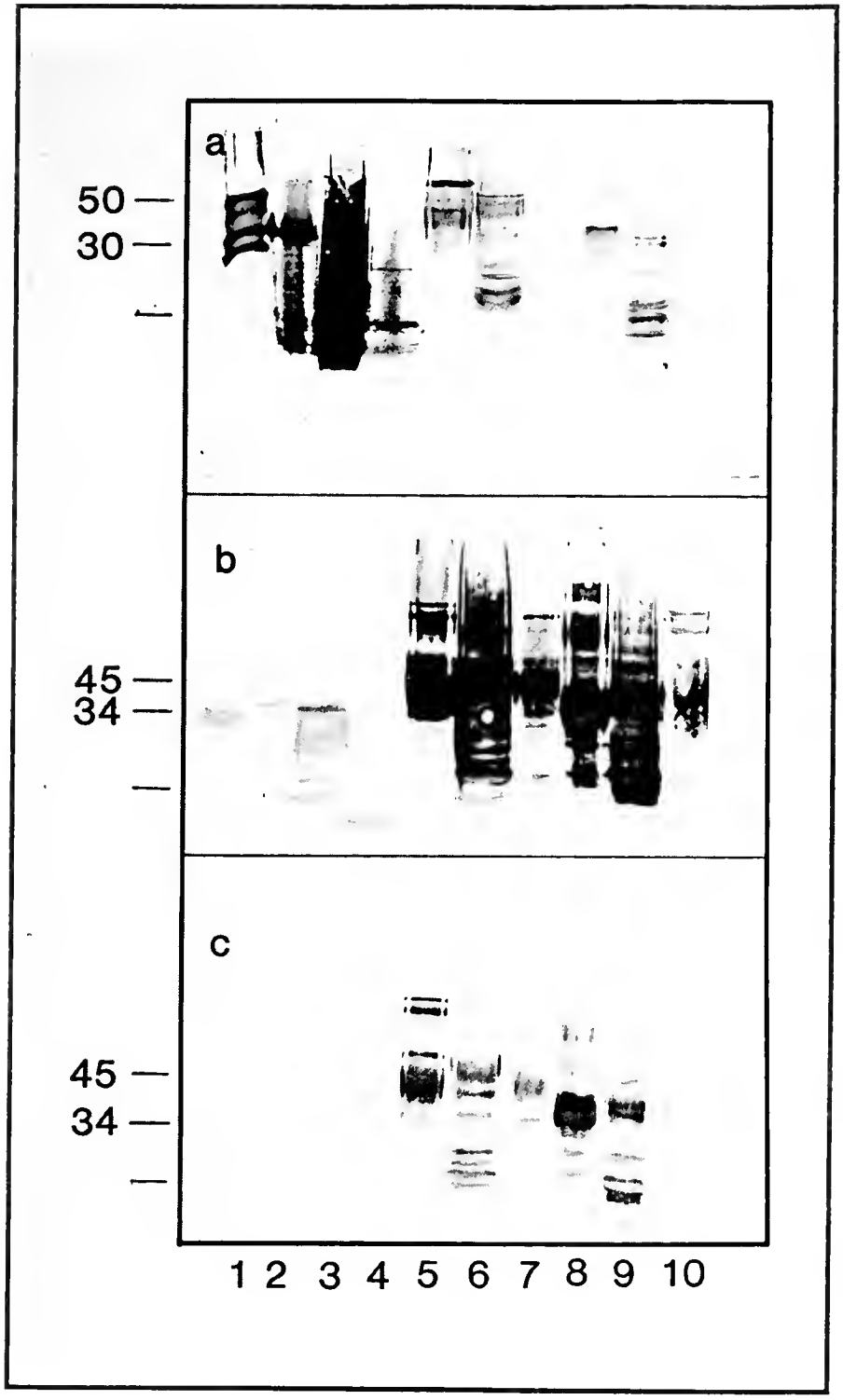
All gels were formatted the same as indicated below. Lane 1: Goat IgG standard; Lane 2: PAP-II standard protein; Lane 3: CNBr digest of PAP-II; Lane 4: Blank; Lane 5: Peak 1(44 kD anti-PAP-II crm); Lane 6: CNBr digest of Peak I ; Lane 7: 0.1 dilution of Peak 1 protein; Lane 8: Peak 2 (34 kD anti-PAP-II crm); Lane 9: CNBr digest of Peak 2; Lane 10: 0.1 dilution of Peak 2 protein.

a.Western blot probed with monospecific goat anti-PAP-II (1:250 dilution).

b. Western blot probed with polyclonal chicken anti-Peak 2 (1:2500 dilution).

c. Western blot probed with polyclonal chicken anti-Peak 1 (1:2000 dilution).

d. Silver stained gel formatted as described above.


a
b
c
M.W.
kD
50
30
45
34
45
34
1 2 3 4 5 6 7 8 9 10

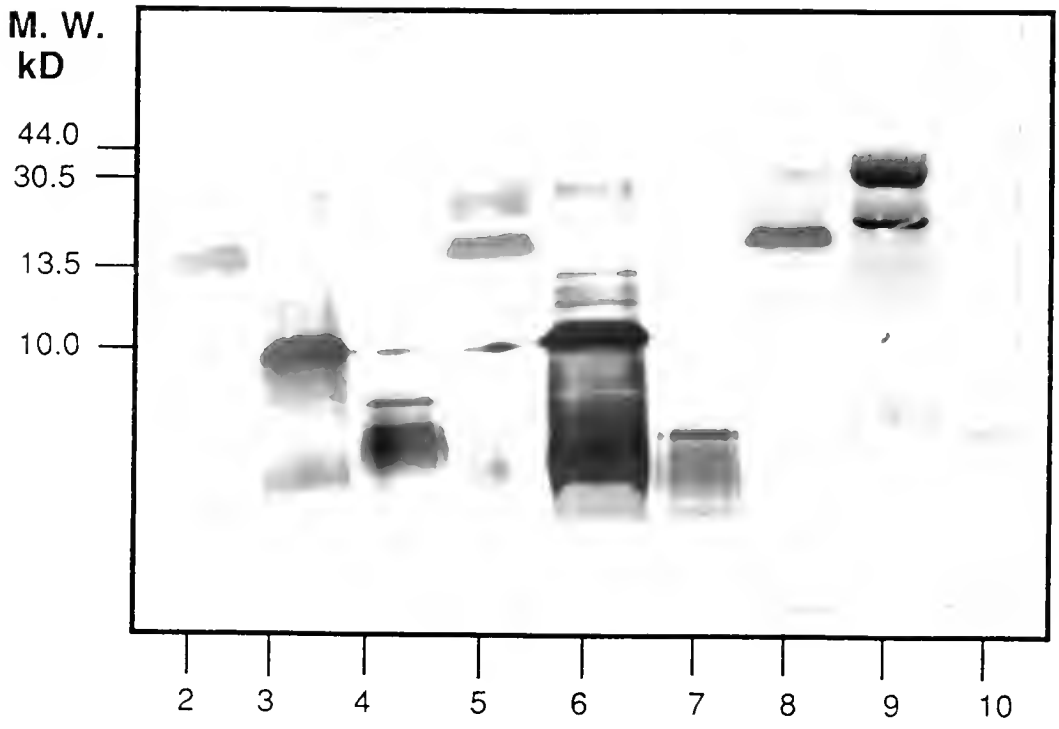


Figure 3.11 (continued)

Table 3.4. Summary of reactivities of major CNBr derived polypeptides with anti-PAP-II, and antibodies against the PAP-II like materials from callus tissue culture of P. rigida.

Peptide Source	Panel and M_r	Lane	Reactivity anti-PAP-II	anti-Peak 1	anti-Peak 2
PAP-II	30.5	2/b	+	-	-/+
PAP-II CNBr					
	7.5	3/b	+	-	
	9.8	3/b	+	-	+
	11.0	3/b	+	-	
	12.8	3/b	+	-	+
	15.5	3/b			
Peak-2	34.0	8/c	+	+	+
Peak-2 CNBr					
	9.8	9/c	+	+	+
	10.5	9/c	+	+	-/+
	13.5	9/c	+	++	++
	19	9/c	-	++	++
	21	9/c	-	+/-	-
Peak-I	44.0	5/d	+	+	+
Peak-I CNBr					
	9.8	6/d	-/+	-	+/-
	13.5	6/d	++	++	++
	15.0	6/d	+	+	+
	19.0	6/d	-/+	+	+
	30.0	6/d	-/+	-/+	-

derived from that protein. Major reactive peptides occur at positions corresponding to molecular weights of 19kD, 13.5kD and small fragments below this.

The detection of peptides with antibody to the Peak 1 (44 kD) protein is shown in panel c of this Figure. This antibody does not detect PAP-II nor PAP-II CNBr fragments after western blotting (Lanes 2-4) but does detect the native antigen (Lane 5). This antibody detects major CNBr fragments (Lane 6) at positions of 30 kD, 19 kD, 15 kD and 13.5 kD. This antibody cross reacts with the Peak 2 protein (Lane 8) and detects major fragments at positions corresponding to molecular weights of 21 kD, 19 kD, 13.5 kD and several fragments smaller than this.

Discussion

The antibody preparations described in the previous chapter, both monospecific and cross-reactive have been utilized in this study to determine which particular PAP proteins are synthesized by pokeweed in callus culture.

Here, P. rigida callus maintained on a modified M-S medium is shown to be capable of synthesizing proteins which share antigenic determinants with the pokeweed antiviral proteins PAP-I and PAP-II isolated from leaf tissue. Using monospecific antibody directed against the seed form of PAP (PAP-S), no cross- reactive compounds were detected, indicating that tissue specific gene expression occurs in this undifferentiated callus, and conforming to the serological data presented previously in Chapter 2. Both PAP-I and PAP-II-like proteins isolated from callus culture by conventional chromatographic techniques are cross-reactive with antibodies prepared against the proteins from spring and summer leaves. This indicates that the

antigenic determinants on the proteins isolated ex planta are shared by the proteins isolated from callus. The fact that a large number of independently growing calli were utilized for the isolation of these proteins argues against these proteins being the product of a single clonal mutant, and suggests rather that they are produced during the normal metabolic activity of these cells.

The ability of tissue culture cells to synthesize two discrete gene products, i.e., PAP-I and PAP-II, as determined by the reactivity to the antibodies used as probes in the present work (as well as the sequence data for PAP-I and PAP-II from leaf tissue, see Table 1.3, Chapter I) indicates that the genes for these proteins are expressed simultaneously and that a temporal sequence of transcriptional and translational events is not requisite for the synthesis of PAP-II. Of interest is the presence of glycosylated forms of the anti-PAP-II crm and the higher molecular weight forms of the anti-PAP-I crm which also may be glycosylated. The glycosylation of proteins is thought to play a role in the targetting of proteins in mammalian and plant cells (Marshall, 1972; Chrispeels, 1984) and the glycosylated species described here could represent intermediates in a processing sequence which has been interrupted by callus induction.

The biosynthesis of the N-linked oligosaccharides of plant and animal cells proceeds by the dolichol-phospate pathway with the key intermediate $Glc_3Man_9GlcNac_2$-PPDol transferring the oligosaccharide to the the protein (Hori et al., 1982; Lehle, 1981; Staneloni et al., 1980). The crude characterization of the carbohydrate moiety on the PAP-II like glycoproteins described here found large amounts of glucose associated with these proteins (see Table 3.1). Even if during biosynthesis the transferred intermediates were not trimmed, one would expect a three-fold molar excess

of mannose over glucose if biosynthesis were blocked at this step. This is not observed here, and makes the presence of such large amounts of glucose associated with the glycoprotein difficult to interpret. The recent experiments described by Rip et al. (1988) may go some way towards resolving this anomaly. Those authors found that the capacity for the formation of Dol-P-Glc exceeds the capacity for the early biosynthesis of either Dol-P-Man or Dol-P-GlcNac and surmise that this may be of some regulatory significance. The large amounts of glucose found associated with the glycoproteins described here may represent a block in the biosynthetic pathways utilizing Dol-P-Glc resulting in an anomalous glycosylation pattern.

Woo and Kang (1976) reported the induction of callus from stem tissue of P. americana but did not report on the presence of the PAP proteins. More recently, Barbieri et al. (1989) have demonstrated that callus from leaf tissue of P. americana is capable of synthesizing a PAP-I like protein (designated PAP-C) but did not report the presence of PAP-II. Their PAP-C may be identical to the protein I identified as anti-PAP-I crm which is a major component of Pool 1 (see Figure 3.5 a).

The identity of the tissue culture PAP-like proteins with the proteins from leaf or seed tissue was investigated utilizing chemical hydrolysis by CNBr and immunological criteria (reactivity of peptide fragments in Western blot analyses) in the previously described experiments. The utilization of protein fragmentation and comparison of the derived polypeptides with a protein of known origin has benifited the elucidation of shared epitopes. Analysis of proteins by SDS-PAGE following cyanogen bromide digestion is frequently useful to establish homologies between proteins presumed to be related, and small peptide fragments may be blotted and sequenced to define

homologies. Cyanogen bromide cleaves at methionine, and is highly specific for this amino acid, although cysteines may be slowly oxidized. Total amino acid compositional analysis can give quantitative information on the amount of methionine in a protein. From these data the expected number of fragments from a CNBr digest may be predicted. Because of the great specificity of CNBr cleavage for methionine, this reagent has been utilized widely for structural elucidations of proteins. The earliest structural studies were carried out on bovine pancreatic ribonuclease and chymotrypsin, but more recently, Asano et al. (1986) have utilized the CNBr mediated cleavage of the barley protein inhibitor in conjunction with enzymatic proteolysis to determine the complete primary structure of that protein.

The amino acid compositions of the PAP proteins have been reported. PAP-I is reported to have 4 methionines (Irvin et al., 1980), PAP-II, 8 methionines (Irvin et al., 1980) and PAP-S, 6 methionines (Barbieri et al., 1982). There is however, little information in the literature dealing with the fragmentation of these RIPs by CNBr. The isolation of biologically active fragments of a complex protein after proteolytic cleavage or CNBr fragmentation is an important extension of this methodology. The application of CNBr to this end was described by Lugnier et al. (1974) who reported the isolation of a peptide from ricin after tryptic digest which was inhibitory in an in vitro translation system.

From the SDS-PAGE data presented in Figure 3.10 it is clear that the PAP proteins from leaf and seed tissue share few, if any CNBr generated peptides in common. These data are in general support of the interpretation of these proteins as separate gene products.

The analysis with monospecific and cross-reactive antibody probes presented in Figures 3.11 a, b and c indicates that there are immunoreactive fragments shared between the anti-PAP-II crms and the leaf form of PAP-II. In addition, all of the proteins are susceptible to attack by chymotrypsin (data not shown) which suggests that this reagent could be used effectively for epitope mapping studies of the PAP proteins. Such an approach has been initiated with the related RIP, trichosanthin, by Ke et al. (1988) who have undertaken to identify epitopes on trichosanthin utilizing chymotrypsin derived fragments.

While there is a suggestion in the literature that the ribosomes of the pokeweed are insensitive to the action of the PAP proteins (Batelli et al.,1984), this point is not definitively proven. The proteins isolated from tissue culture do have inhibitory activity towards in vitro translation systems as shown in Table 3.3. However, this activity is reduced from that of the green leaf forms of PAP-I and PAP-II by one or more orders of magnitude. These data are supportive of a protective role for the glycosylation of these putative intermediates. Glycosylation could provide a protective role by diminishing the known ribosome-inactivating properties of these proteins during synthesis and intracellular trafficking. Such modifications may also provide for compartmentalization during the transit from the point of synthesis to the final site of sequestration. If so, the compartmentalization of these proteins may afford the required protection following biosynthesis. Compartmentation of the endogenous toxic protein is a strategy adopted by castor bean (the source of ricin) for protecting cytoplasmic ribosomes from inactivation during germination and early growth. The ricin is strictly compartmentalized in vacuoles in the endosperm during germination (Harley and Beevers, 1984; Harley and Beevers, 1982).

Gold label immunocytochemistry on thin sections from callus tissue has suggested such a compartamentalization (see Figure 3.9). The anti-PAP-II crm appears to be associated with undefined elements in the cytosol (vesicle fractions ?) and the anti-PAP-I crm to be wall bound . The differential distribution of these two protein species supports the interpretation that the PAP-I like material is correctly synthesized and processed to be localized in the wall (see Ready et al., 1986) and that the PAP-II like material may exist in a sequestered form as intermediates in the cytoplasm. The fact that the proteins isolated from mature leaf tissue in vivo are devoid of detectable carbohydrate suggests that there is rapid processing of the glycosylated forms to yield the forms routinely isolated from leaf tissue. That there is some observed processing of the PAP-II like glycosylated forms in callus is indicated by the variability in the yield of the 44 kD glycosylated form from different preparations, with a relative increase in the percent of the 34 kD form at the apparent expense of the 44 kD form (unpublished observation). It will now be of interest to determine if analogous forms can be identified in different tissues in vivo as a function of plant development.

CHAPTER IV
EXAMINATION OF THE TISSUE CULTURE DERIVED ANTI-PAP-II CROSS-REACTIVE MATERIALS WITH AN ANTIBODY PROBE DIRECTED AGAINST A UNIQUE AMINO TERMINAL SEQUENCE ASSOCIATED WITH THE TISSUE CULTURE PROTEINS

Introduction

As described in the previous chapter, investigations of the PAP-II like proteins obtained from a callus tisssue culture system revealed a unique amino terminal sequence associated with those proteins. The sequence was sufficiently distinctive that I speculated that it was involved in the targetting or processing of these proteins in the tissue culture system. Very little information exists on the biosynthesis of the RIPs. To examine the potential role of this sequence in the biosynthesis of the PAP proteins, this unique amino acid sequence was synthesized as a peptide of 18 amino acids and antibodies were prepared against this sequence. The antibodies were affinity purified and utilized to screen crude extracts of leaf tissue for protein(s) bearing this antigenic determinant. This chapter describes the results of those experiments and details the characterization of the antibody prepared against this peptide.

Materials and Methods

Sequence characterization and synthetic peptide preparation

Amino terminal sequence data was obtained from purified tissue culture proteins which were shown to be cross-reactive with antibody to PAP-II. These purified proteins (shown to be homogeneous by SDS-PAGE and

EITB, see Chapter 3) were dialyzed against deionized distilled water and lyophilized. The lyophilized powder was supplied to the Protein Chemistry Core Facility, ICBR, Univ. Fla., for automated amino terminal sequencing. Alternatively, the protein was blotted to PVDF membranes and supplied to the same facility. The two proteins, identified as Peak 1 (44 kD) and Peak 2 (34 kD) yielded the following amino terminal sequence (concensus): Ser His His His Arg Cys His His His Arg Pro His His His Arg Pro Gly. This sequence was then synthesized in the same facility (utilizing an Applied Biosystems Peptide Synthesizer, Model 430A which utilizes the Merrifield technology of t-Boc resins) with a lysine in the first position, followed by the sequence as given.

Preparation of peptide-BSA conjugate and antibody

The 18 amino acid peptide was coupled to BSA via glutaraldehyde according to the procedure of Reichlin (1980). The conditions for the conjugation were such that 5 moles of peptide were reacted per mole of BSA. Two separate conjugations were prepared and the products, following dialysis against 0.1 M Tris-HCl, 0.05 M NaCl, pH7.6 (Tris-buffered-saline, TBS) were chromatographed on a P-6 (Bio-Rad) column in the same buffer. The protein peak (determined by A_{280}) from this chromatographic step was pooled, and stored at $4^{o}C$. For the preparation of antibody, peptide conjugate and free peptide were injected separately into white leghorn chickens. One mL (as a 0.1 mg/mL solution) was injected into chickens for the preparation of antibody. For comparative purposes, unconjugated peptide as a 4.0 mg/mL solution in TBS was injected directly into a chicken. The antibody responses of the three chickens were determined by ELISA analysis of the egg-yolks at 3 day intervals post injection. The chickens were boosted at 9 days by injecting the same amount of material. Eggs from

a single chicken which showed a positive reponse by ELISA to the free peptide were pooled and the IgY fraction was prepared according to the methodology of Polson et al. (1985). The pooled, purified IgY was applied to an affinity column (Affigel-10 coupled to peptide) in TBS and after extensive washing, bound antibody was eluted with 0.3 M NaCl, pH 11.2 (pH adjusted by the dropwise addition of NH_4OH). Fractions of 1.0 mL were collected. Antibody protein was identified as A_{280} in elution buffer and tubes containing eluted antibody protein were pooled and dialyzed against TBS. This pooled, affinity purified antibody was utilized in western blot analyses of PAP-like proteins from callus and from leaf tissue. Elution with 0.1 M glycine (pH 2.3) was attempted but failed to elute specific antibody.

ELISA

ELISA analysis was performed as described in previous chapters. For the screening of IgY antibody against peptide conjugate or free peptide, free peptide was routinely plated as 100 uL aliquots in carbonate buffer as serial twofold dilutions from an initial stock of 5.0 ug/mL. This resulted in a range of antigen from 500 ng to 1.5 ng per well. Primary antibody was delivered as dilutions in PBS: Tw. Secondary antibody was a commercially obtained (Sigma) rabbit anti-chicken IgY conjugated to alkaline phosphatase.

EITB

EITB analysis was performed as described previously. For the analysis of detection of peptide by chicken antibody, BSA-peptide conjugate was electrophoresed and trans-blotted as free peptide was not retained in the separating gel due to its small size (see Chapter 2).

Results

Characterization of antibody to peptide conjugate and purification by affinity chromatography

The results of the ELISA analysis for the antibody production in yolks of immunized eggs is presented in Figure 4.1. This Figure shows the response 10 days following boost of two chickens (59-2 and 59-3) injected with separate peptide-BSA conjugates and that of the chicken (59-4) injected with free (unconjugated) peptide . From the data, it is clear that all chickens mounted an immune response to the peptide antigen, whether immunized with a BSA peptide conjugate or with the free peptide. In all cases the ELISA titers were determined with free peptide as antigen. Those yolks which showed a titer by ELISA were pooled and processed as described by Polson et al. (1985). The resulting preparation, enriched for IgY, was affinity purified by chromatography on an Affi-Gel 10-peptide column. The results of the affinity purification for one trial (59-3) are shown in Figure 4.2 This Figure demonstrates the elution profile (Figure 4.2 a) and the ELISA data (Figure 4.2 b) obtained from the preparation of affinity purified antibody from chicken 59-3. The arrow in Figure 4.2 indicates the point of application of the elution buffer, pH 11.0. It is of interest that the greater proportion of this antibody preparation did not bind to this affinity column as indicated by the large amount of antibody (as measured by A_{280}) present in the wash through (fractions 4-20). Specific antibody eluted with pH 11.0 buffer (present in fractions 22-29) represented less than 1% of the total protein (A_{280}) applied to the column. The material in these fractions (22-29) was pooled and dialyzed against TBS. The reactivity by ELISA of this affinity purified antibody (at a 1:100 dilution in PBS:Tw) after dialysis, to free

Figure 4.1. ELISA analysis of chicken IgY antibody to peptide-BSA conjugate and free peptide.

Free peptide and conjugate were prepared as described in the text and injected into white leghorn chickens. The chickens received a boost at day 10. Eggs were collected daily. The data represent the titer of egg yolks 10 days post-boost processed as described in the text. Free peptide was plated as antigen at 30, 60, 125 and 250 ng/well in 100 uL aliquots. The ELISA plate was processed as described. Three dilutions of antibody were assayed for each trial. For each graph the antibody dilutions are as follows:

antibody dilution,1:1000

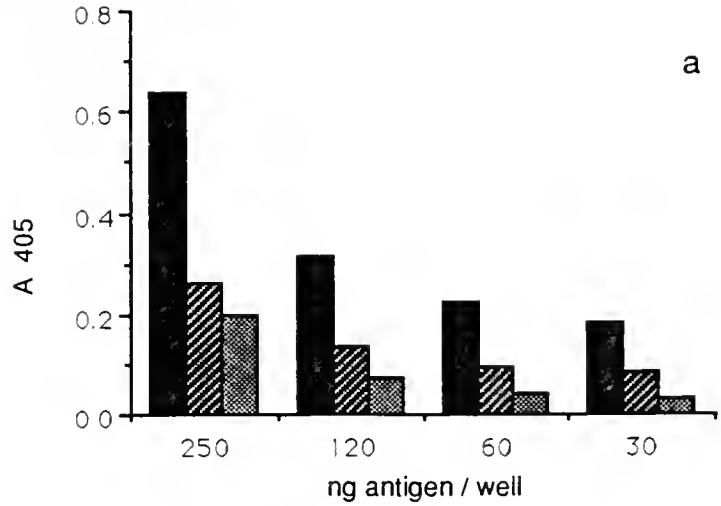
antibody dilution,1:2000

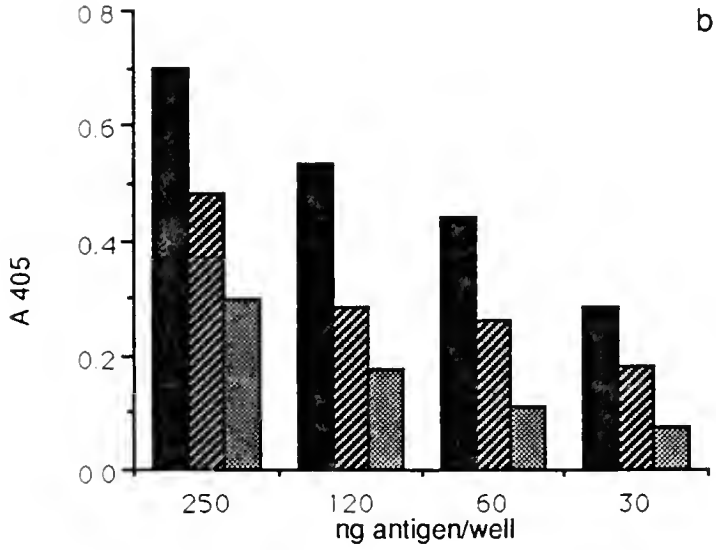
antibody dilution,1:4000

a. Titer of of egg yolk IgY obtained from chicken 58-2 injected with peptide-BSA conjugate when measured by ELISA with free peptide as antigen.

b. Titer of of egg yolk IgY obtained from chicken 58-3 injected with peptide-BSA conjugate when measured by ELISA with free peptide as antigen.

c. Titer of of egg yolk IgY obtained from chicken 58-4 injected with free peptide when measured by ELISA with free peptide as antigen.


a
0.8
0.6
0.4
0.2
0 0
A 405
250
120
60
30
ng antigen / well


b
0 8
0.6
0.4
0.2
0 0
A 405
250
120
60
30
ng antigen/well

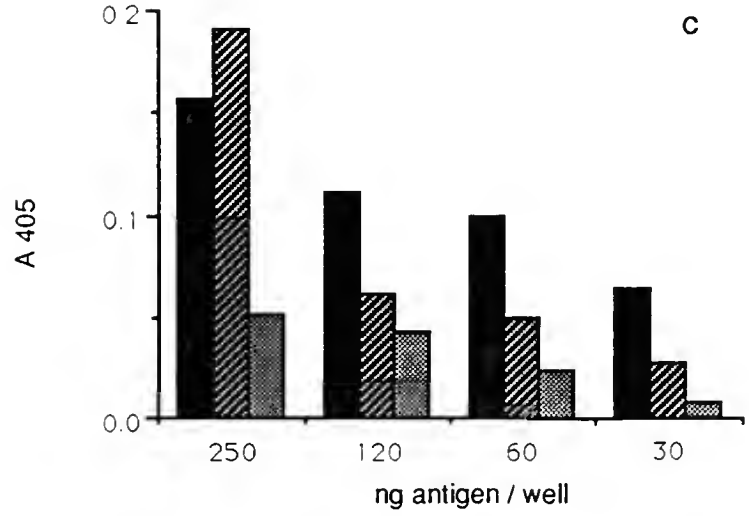
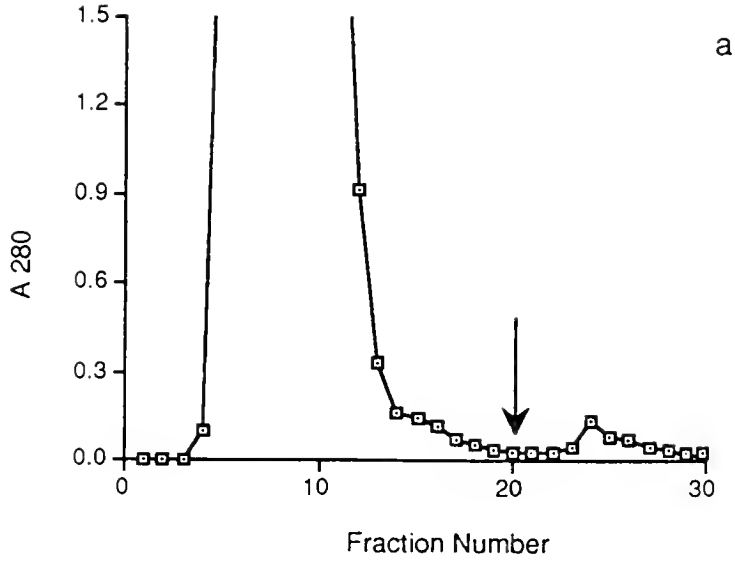
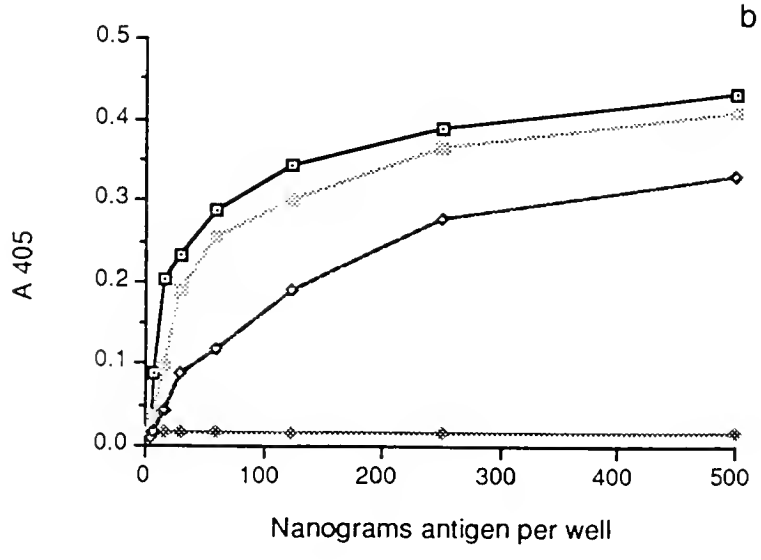

c
0 2
0.1
0.0
A 405
250
120
60
30
ng antigen / well

Figure 4.2. Elution profile of IgY antibody from peptide affinity column and characterization by ELISA of affinity purified anti-peptide.

a. IgY prepared as described from egg yolks of chicken #58-3 was applied to an affinity column composed of peptide coupled to Affi-gel 10. The column was washed extensively with tris-buffered saline following sample application, and specifically bound antibody was eluted with 0.3 M NaCl, pH 11.0. The point of application of this buffer is indicated by the arrow in Figure 4.2 a. Protein containing fractions were measured by following the absorbance at 280 nm.

b. Affinity purified antibody obtained from the affinity chromatography of IgY from chicken 58-3 was assayed by ELISA as described in the text. The ELISA response to free peptide (—⊡—), BSA (—◈—), Peak-1 (44 kD anti-PAP-II crm from tissue culture, —◇—), and Peak-2 (34 kD anti-PAP-II crm from tissue culture, —□—) was determined with an antibody dilution of 1:100.


a
1.5
1.2
0.9
0.6
0.3
0.0
A 280
0
10
20
30
Fraction Number


b
0.5
0.4
0.3
0.2
0.1
0.0
A 405
0
100
200
300
400
500
Nanograms antigen per well

peptide, Peak 1 protein (44 kD anti-PAP-II crm from callus tissue), Peak 2 protein (34 kD anti-PAP-II crm from callus tissue) and to BSA is shown in Figure 4.2 b. The antibody is non-reactive with BSA present in amounts ranging from 3.75 to 500 nanograms. The reactivity of the free peptide is comparable to that of the 34 kD anti-PAP-II crm from tissue culture, while the reactivity of the 44 kD anti-PAP -II crm is about one-half that of the free peptide.

EITB analysis

The ability of this affinity purified antibody to detect specific proteins following transfer to nitrocellulose is shown in Figure 4.3. This Figure (4.3 a) demonstrates that this antibody detects the Pool 2 material from CM-52 (containing anti-PAP-II crm, Lane 1), the fraction from SG-75 chromatography of Pool 2 designated Peak 1 protein (44 kD, Lane 2), and Peak 2 protein (35 kD , lane 3) and the peptide-BSA conjugate (Lane 4). Figure 4.3 b shows the appearance of the proteins when stained with silver in the polyacrylamide gel. The diffuse nature of these polypeptides is apparent from this Figure. In addition, it appears as though the material in Peak 2 is beginning to degrade on storage, resulting in the appearance of several peptides in the silver stained gel (4.3 b, Lane 3).

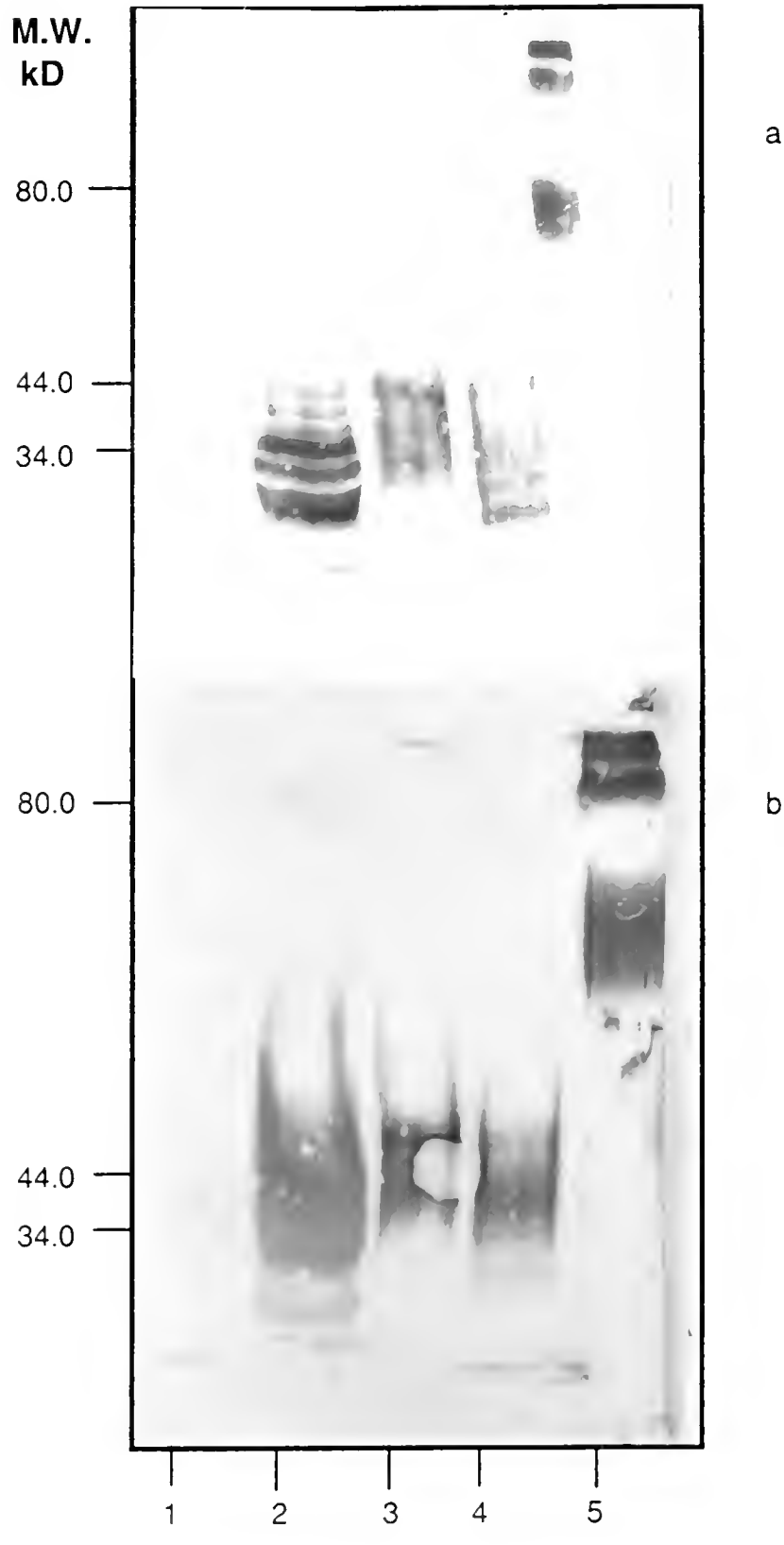
The ability of this antibody to serve as a probe of leaf tisssue for the presence of PAP related proteins bearing this same antigenic sequence is demonstrated in Figure 4.4. This Figure demonstrates by Western blot analysis the reactivities of crude extracts obtained from P. rigida leaf tissue at a number of different time points (March -December) to the affinity purified antibody described above. The data demonstrate that this antibody is capable of detecting polypeptides with molecular weights of 33 kD and 28 kD (see arrows, Figure 4.4) present in crude extracts . These peptides are

Figure 4.3. Western blot analysis of the ability of anti-peptide conjugate to bind to the anti-PAP-II crms and the conjugated peptide.

SDS-PAGE analysis of the 44 kD and the 34 kD protein from tissue culture of P. rigida and the peptide conjugate was performed as described on 12% polyacrylamide gels. Silver staining of gels was performed according to the method of Wray et al. (1981). Peptide conjugate was prepared as described from the glutaraldehyde mediated coupling of BSA to free peptide. Tissue culture proteins were purified as described by CM-52 chromatography and gel sieving. Proteins were transferred to NC-42 nitrocellulose for one h at 0.4 amp. Western blots were processed as described. Affinity purified chicken anti-peptide conjugate was applied to the blot at a dilution of 1:100.

a. Silver stained polyacrylamide gel showing the appearance of the proteins after SDS-PAGE. Lane 1: molecular weight markers; Lane 2: Pool 2 concentrate from CM-52 prior to SG-75 gel sieving chromatography, 25 uL; Lane 3: Peak 1 protein (44 kD anti-PAP-II crm), 0.5 ug; Lane 4: Peak 2 protein (34 kD anti-PAP-II crm), 0.5 ug; Lane 5: peptide conjugate, 0.5 ug

b. Western blot of gel described in a (above). The blot was probed with chicken anti-peptide conjugate at a dilution of 1:100.


M.W.
kD
80.0
44.0
34.0
a
80.0
44.0
34.0
b
1
2
3
4
5

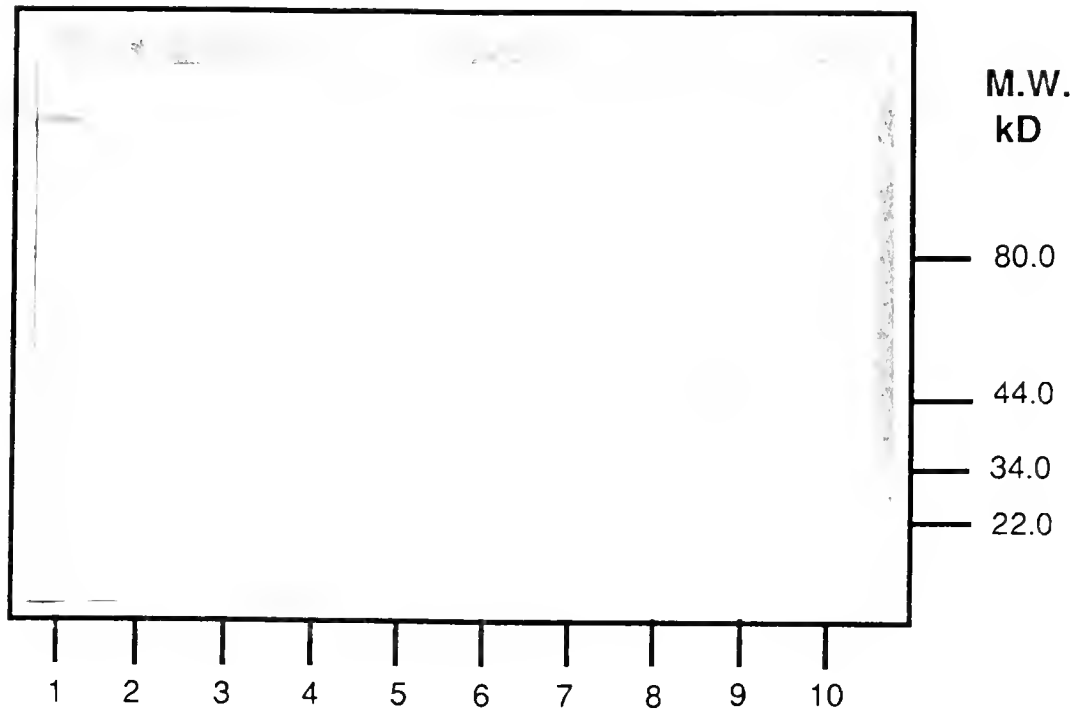


Figure 4.4. Western blot demonstrating the ability of anti-peptide conjugate to detect discrete protein entities in crude extracts from leaf tissue of P. rigida.

Crude extracts obtained from leaf tissue of P. rigida at seven time points between March and December were electrophoresed and transferred to nitrocellulose for western blot analysis as described. The samples correspond to the NS samples (1-7) discussed previously (see Chapter II, Figures 2.10, 2.11, 2.12). The western blot was probed with affinity purified anti-peptide conjugate at a1:100 dilution.

Lane 1: Peptide conjugate, 0.5 ug; Lane 2: NS-1 (3/8), 25 uL; Lane 3: NS-2 (4/10), 25 uL; Lane 4: NS-3 (5/2), 25 uL; ,Lane 5: NS-4 (6/4), 25 uL; Lane 6: NS-5 (7/2), 25 uL; Lane 7: NS-6 (8/22), 25 uL; Lane 8: NS-7 (12/5), 25 uL; Lane 9: Peak 1 (44 kD anti-PAP-II crm), 0.5 ug; Lane 10: Peak 2 (34 kD anti-PAP-II crm), 0.5 ug.

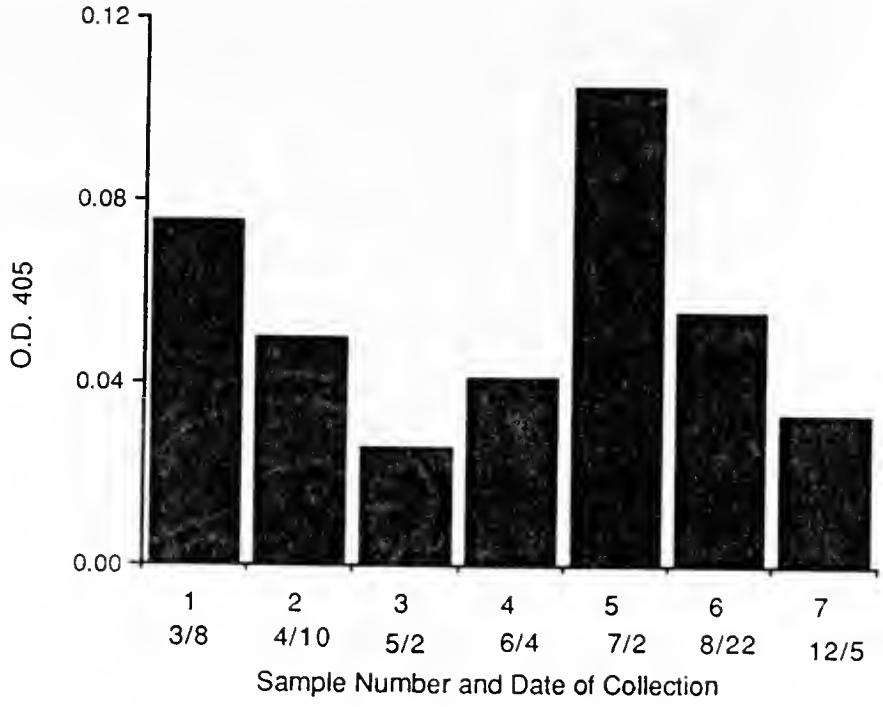


Figure 4.5. ELISA reactivity of affinity purified anti-peptide with crude extracts from leaf tissue of P. rigida obtained at intervals between March and December.

ELISA analysis was performed on crude extracts from leaf tissue of P. rigida obtained from plants sampled at the seven time points noted. Crude extract was prepared as described by heating buffer extracted leaf tissue to 68°C, cooling and centrifuging. Extracts were plated as 1:200 dilutions in carbonate buffer (100 uL/well). Protein content of samples was measured by the BCA method as described in the text. Affinity purified antibody, obtained as described (see Figure 4.2) was applied as a 1:100 dilution in PBS:Tw . The data are presented as O.D. 405/ug protein plated.

detected equally in samples from March- June (Lanes 2-5, Figure 4.4). Lane 5, corresponding to July extract is empty, as though the sample were never applied to the gel. Lanes 6 (August) and 7 (December) show the presence of these same two reactive entities, but also the appearance of higher molecular weight species reactive with this antibody. The peptide at 28 kD detected in these crude extracts is also present in the Peak 2 (34 kD anti-PAP-II crm, Lane 9) and may represent a peptide derived by autodegradation of this sample.

ELISA data related to Figure 4.4 are presented in Figure 4.5. In this Figure, the reactivity of the crude extracts analysed by western blotting is determined by ELISA. Affinity purified antibody was used at a dilution of 1:100, and crude extracts were plated as 1:200 fold dilutions in carbonate buffer. The data are corrected for the amount of protein in each extract by dividing the observed O.D. 405 by the total amount of protein assayed in the ELISA. The reactivity of the later samples, lanes 4-6, clearly increases and then decreases. This increased reactivity in the later samples may relate to an increased presence of immuno-reactive species bearing this unique sequence as an antigenic site.

Discussion

The very unique amino terminal sequence identified from the PAP-II like proteins from callus tissue may represent a transit peptide associated with the targetting of these proteins in tissue culture. This peptide sequence is uniquely associated with anti-PAP-II crm and not with the anti-PAP-I crm. Table 4.1 presents the amino terminal sequence for the tissue culture derived proteins described in the present study, and for comparative purposes the amino terminal sequence data derived from the 5'- nucleic acid sequence for ricin and saporin -6 (SO-6). These 'leader' sequences for

the ricin and saporin may be involved in the targetting/processing of these RIPs. Examination of the sequence data presented in Table 4.1 reveals no obvious homologies between the two RIPs for which nucleic acid sequence data are available, and none with the unusual amino terminal sequence found associated with the the PAP-II like proteins from tissue culture.

Table 4.1. Published amino terminal amino acid sequences from ricin, saporin-6 and P. rigida tissue culture proteins which may correspond to 'leader' sequences.

RIP	'LEADER' SEQUENCES
RICIN[a]	MYAVATWLCFGSTSGWSFTLEDNN
SO-6[b]	MKIYVVATIAWILLQFSAWTITDAV-
Peak 2 Callus[c]	SHHHRCHHHRPHHHLYACFHLPPI
Peak 1 Callus[c]	AHRCHHHRHR

a. Lamb et al., 1985
b. Benatti et al., 1989
c. Ervin, 1989 (this work)

The antibodies made against the peptide described here are capable of binding specifically to the two PAP-II like proteins from tissue culture (Peak 1 and Peak 2) on Western blots (see Figure 4.3, Lanes 2 and 3). More importantly, the antibody appears to recognize high and low molecular weight components present in crude extracts from leaf tissue of P. rigida after SDS-PAGE and Western blotting (see Figure 4.4). This binding is not uniform across all time points, but rather is associated with a distinct time interval which may correspond to a period of processing events related to the biosynthesis of the PAP-II proteins. The binding of this antibody to either

the native antigen (from which the peptide sequence was derived) or to protein(?) species present in crude extracts results in a weak signal in the western blot analysis. This observation is not unexpected for antibody directed against small immunogenic peptides derived from larger molecular weight proteins (McCray and Werner, 1987).

The composition of this peptide is highly distinctive and was considered to be potentially involved in targetting of the PAP-II proteins. Histidine rich glycoproteins are found variously distributed throughout Nature. Those described from plants often are found associated with structural protein elements of the cell wall. In addition, and probably unrelated to the observation reported here is a histidine rich glycoprotein associated with human platelets , and a tripeptide (which shows the conserved sequence ala-his-his) from a peptide found in Plasmodium falciparum . A histidine rich protein (73% histidine) is encoded by the species P. lophurae . Note that while these examples lie well outside the plant kingdom, they indicate that eukaryotes may and do encode proteins which show a high frequency of histidine, and the presence of the histidine may be related to the function of the protein.

The motif of the peptide, if not the exact sequence, is reminiscent of the hydroxyproline rich glycoproteins (HRGP's) associated with wall tissue of higher plants. The major species of wall HRGP's identified to date fall into three main classes. 1) Haemagglutinating glycoproteins associated with the Solanaceae which are involved in the 'wounding ' response, 2) acidic glycoproteins (rich in serine, alanine and hydroxyproline) which may function in cell-cell recognition and 3) the extensins (rich in hydroxyproline, serine, tyrosine and lysine), which are presumed structural components of the primary cell wall. A conserved repetitive sequence of Ser -$(Hyp)_4$ is

present in all extensins examined. Similar repetitive sequences have been described from Chlamydomonas reinhardtii, for which arginine appears to be the major basic amino acid. Non-HRGP wall proteins have been described in which a repetitive motif of X-Y-Pro-Pro or Gly-X is present (Cassab and Varner, 1988). All of these proteins may be structural in the wall, but they have also been implicated to increase following wounding , ethylene treatment, or following fungal attack (Showalter and Varner, 1987). The exact nature of the role for any of these proteins needs to be elucidated. However, it is clear that these proteins must be targetted correctly to the wall for incorporation during growth, or secondarily, following wounding or fungal invasion. As thus, they may serve as useful models for understanding the signals necessary to successfully target proteins to the wall. However, the exact mechanisms by which any plant protein is incorporated into the texture of the plant cell wall remain obscure.

The presence of this basic, histidine rich amino terminal sequence on the PAP-II like proteins from tissue culture may relate to their function in the wall, or their targetting to the wall. It may indicate an as yet untested role for these proteins as wall structural components, which have secondarily acquired an enzymatic activity. In either case, this peptide and the associated antibody will provide a uniquely intriguing window through which to view the intracellular targetting and processsing of these PAP-like proteins.

CHAPTER V
CONCLUSIONS

The previous chapters have detailed the results of my investigations on the pokeweed antiviral proteins from the common 'Southern' pokeweed, P. rigida. These investigations were undertaken to examine the immunological basis for the cross-reactivity observed for these proteins, and to develop specific antibody probes for these proteins in plant tissue. A callus tissue induced from leaf tissue was shown to possess proteins which were cross-reactive with antibodies developed against the leaf tissue proteins. This analysis allowed the identification of protein species with molecular weights greater than 30 kD which were cross- reactive with anti-PAP-I, and a discrete protein moiety with an apparent molecular weight of 29.5 kD, identical to PAP-I from leaf tissue. When this same callus tissue was assayed for the presence of PAP-II, no discrete protein species were identified with a molecular weight identical to that of PAP-II (30.5 kD). Rather, two protein species were identified with relative mobilities corresponding to molecular weights of 44 kD and 35 kD. These anti-PAP-II cross-reactive proteins were shown to be glycosylated, with a large amount of glucose as the major carbohydrate. These anti-PAP-II cross-reactive proteins were isolated and purified to homogeneity for amino terminal sequencing. Both glycoproteins were shown to possess a unique NH_2-terminal sequence rich in positively charged amino acids. This sequence was synthesized, and the peptide was conjugated to BSA for the

preparation of antibody. Monsospecific antibody against this unique amino terminal sequence was prepared by affinity chromatography and was utilized as a probe of leaf tissue to identify immuno-reactive protein species present in crude leaf extracts bearing this same antigenic sequence. The identification of glycoproteins from tissue culture (anti-PAP-II crms) bearing a unique amino terminal sequence, and the ability of antibody against this peptide to detect discrete high molecular weight forms in leaf tissue extracts are presumptive evidence for these proteins being intermediates in the processing of the PAP- like proteins in tissue culture. While this research was in progress, a report appeared describing the isolation of a PAP-I like protein from callus tissue of P. americana (Batelli et al., 1989). The authors did not report the isolation of a PAP-II like protein, but their report on the presence of PAP-I lends support to the observations reported here, and confirms the utility of the tissue culture system for examining biosynthetic events. While we were unable to address directly questions pertinent to an understanding of the regulation of the expression of these proteins at the transcriptional or translational level, the antibodies characterized in this study and the proteins we have isolated and sequenced from tissue culture should provide a solid base for such studies. In particular, the ability of the antibody against the amino terminal sequence to detect discrete peptide entities in Western blots of crude leaf extracts (Figure 4.3) provides a convincing link between the proteins synthesized in the tissue culture system in vitro and the plant system in vivo.

Much of the data presented herein depends on the cross-reactivities observed between these PAP proteins in Western blots. Any model which is invoked to explain cross-reactivity at the level of protein structure obtained from widely variant tissues as callus, seed and leaf must assume shared

eptiopes, either continuous or discontinuous. It is of interest that monospecific anti-PAP-II is capable of recognizing a large number of 'antigens' in crude extracts of leaf tissue, while anti-PAP-I reacts only with the PAP-I antigen present in these extracts (see Figure 2.7, Chapter 2). Monospecific anti-PAP-S is weakly reactive with PAP-I in Western blots, but not by ELISA. Thus , PAP-II may represent a protein species which carries 'universal' epitopes related to its function or localization. The antibody probes developed in the course of this research have allowed for studies on the localization of the PAP-like proteins in the tissue culture system. Evidence for the differential localization of the tissue culture proteins in callus tissue was obtained, which might support the inference of different roles for these proteins, with PAP-I serving as a primary barrier, and PAP-II as a 'suicide' or secondary barrier within the cytosol. Apart from their suggested function as defensive agents, other possible roles of these proteins within the plant have been posited. These are summarized below in Table 5.1.

It is evident that the unique ability of these proteins to effect the specific inactivation of the ribosomal RNA underlies all of the postulates concerning the function of these proteins in vivo. However, other features of these proteins, in particular their very basic pI's, offer the possibility that these proteins are involved in structural properties of the plant cell wall. It may well be that the enzymatic activity observed in vitro for these proteins is secondary to their functional role in the plant. This inability to correlate a specific activity observed for a plant protein with a function in the plant has a precedent in the haemagglutinating properties of the plant lectins. The ability of the lectins to agglutinate red blood cells in vitro is a convenient

Table 5.1. Possible functions of the ribosome-inactivating -proteins for the plants in which they occur.

1. Primitive immune system
    a. Self-ribosomes are insensitive to the toxin when compared to the sensitivity of evolutionarily distant ribosomes.
    b. Heterograft rejections between unrelated plants.

2. Defense against parasitic invaders.
    a. antiviral
    b. antifungal
    c. antimammalian

3. Regulatory enzyme during development.

4. Structural/regulatory in the wall.

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assay for the presence of those proteins, but is undoubtedly distantly related to their evolved function for the plants in which they occur. Any model which attempts to account for the presence of these proteins in the plants in which they occur must coordinate the enzymatic properties, biophysical properties, developmental regulation, and expression of these proteins. Our understanding of these proteins for a long time has been dominated by their unique and highly specific enzymatic activity. The antibody probes and the approach adopted in this work may afford a new perspective for understanding of these proteins, in relationship both to their molecular structure and their developmental significance. The tissue culture system should provide a convenient cell system to evaluate the antiviral activity of these proteins, and may provide a system optimally designed for the isolation of the genes involved in the synthesis of these proteins.

Over the past years, some observations have appeared in the literature regarding the vegetative storage proteins (VSP) of soybean. These proteins are of interest in that they show a flexibility in their storage capacity, accumulating and decreasing as needed by the plant. VSP accumulates in leaves in vacuoles (Staswick, 1990). Of particular interest is the published DNA sequence for this protein (Staswick, 1988). Beginning at amino acid number 230, there is a sequence with an unusually high preponderance of arginine (R-S-P-R-R-P-Gln-R-R-Lys-...). This sequence is vaguely reminiscent of the sequence we found associated with the tissue culture proteins cross -reactive with anti-PAP-II in that it shows a high frequency of repeated blocks of the basic amino acid, arginine. In addition, the VSP gene shows homology with the soybean seed lectin gene (Le). It may be that all of these proteins, the RIPs, the lectins, VSPs, and seed storage proteins are interrelated.

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BIOGRAPHICAL SKETCH

Sean Edward Ervin was born October 9, 1953, in New Orleans, La. After several years of travelling and working at a number of science related jobs, he obtained his Bachelor of Science degree in biology at the University of California, Santa Cruz campus. He graduated with honors for an undergraduate research project in the laboratory of Kenneth V. Thimann, May, 1979. He subsequently spent one year working in the laboratory of John G. Torrey at the Harvard Forest in Petersham, Mass., before he moved to Gainesville, Fla., in 1980. He earned his Master of Science degree with David H. Hubbell in the Department of Soil Science in 1983. Prior to joining the Department of Microbiology and Cell Science in 1986, Sean worked for three years as a laboratory manager in the Department of Comparative and Experimental Pathology. In his spare time Sean enjoys playing soccer and banjo. He has travelled widely throughout the Americas and speaks several languages. Sean is married with three children.

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

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